The Dissertation Committee for Gwen Ellen Dressing certifies that this is the approved version of the following dissertation:

Membrane progestin receptor expression, signaling and function in reproductive somatic cells of female vertebrates.

Committee:

Peter Thomas, Supervisor

Henrietta Edmonds

Brian Scott Nunez

Izhar Khan

Creagh Breuner

Membrane progestin receptor expression, signaling and function in reproductive somatic cells of female vertebrates.

By

Gwen Ellen Dressing, B.S.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in partial fulfillment

of the requirements

for the degree of

Doctor of Philosophy

University of Texas at Austin

May 2008

Dedication

To my family, without your support and guidance I would not be where I am today.

Acknowledgements

This research was supported by grants from the Dougherty Foundation and the James R. Dougherty, Jr. Foundation, and an E.J. Lund Fellowship in Marine Science.

I would like to thank my supervisor Peter Thomas for his patience and guidance over the last six years. I am also grateful to my committee members Henrietta Edmonds, Brian Scott Nunez, Izhar Kahn and Creagh Breuner. I appreciate each of their contributions and support throughout my degree candacicy. I would like to especially thank my fellow lab mates Chris Tubbs, Margaret Pace, Abby Benninghoff, Håkan Berg, Yefei Pang, Md. Saydur Rahman, Jing Dong and Susan Lawson. You have all been of immeasurable help to me both personally and professionally.

Finally I would like to acknowledge my family: my parents who have supported me unconditionally and instilled in me a love of learning, my sister, who grounds me in reality, and my grandparents whose support has allowed me to turn my aspirations into reality.

Membrane progestin receptor expression, signaling and function in reproductive somatic cells of female vertebrates.

Publication No. _____

Gwen Ellen Dressing, Ph. D.
The University of Texas at Austin, 2008

Supervisor: Peter Thomas

The goal of the current research was to examine the expression, signaling and function of the membrane progestin receptors (mPRs) in the ovarian follicular cells of the Atlantic croaker (*Micropogonias undulatus*) and in human breast cancer cells. Multiple studies have examined the role of mPRs in the germ cells of several vertebrate classes, yet few studies have examined the role of the mPRs in the somatic cells of reproductive tissues. Therefore this research examines the mechanism of mPR action and its function in somatic cells of female reproductive tissues.

Results from studies on the expression, localization and signaling of the mPRα in co-cultures of granulosa and theca cells from the croaker suggest that the mPRα is localized to the plasma membrane of both cell types and that the mPRα is associated with and signals via pertussis toxin-sensitive inhibitory G proteins to decrease intracellular cAMP and activate ERK. In addition, exposure of follicular co-cultures to progestins that activate the mPRα results in a decrease in serum starvation-induced cell death which is

not replicated by progestins which activate the nuclear progestin receptor (nPR), indicating mPR mediation.

Similar studies in two immortalized human breast cancer cell lines, MDA-MB-468 and SKBR3, suggest that the mPRα is also present in the membranes of these cells and signals in human breast cancer cell lines via activation of a pertussis toxin-sensitive G protein to significantly decrease in intracellular cAMP and activate ERK. Progesterone exposure also decreased serum starvation-induced cell death in SKBR3 cells which are nPR positive and in MDA-MB-468 cells which are nPR negative. Synthetic progestins which activate the nPR but not the mPR were ineffective in inhibiting death in either cell type suggesting that the mPR is the mediator of this progestin action.

mPRα, mPRβ and mPRγ expression analysis of paired normal and malignant breast tissue biopsies from thirteen women revealed that at least one mPR isoform was upregulated in the malignant tissue of 70% of the women. In addition the expression of mPRγ was positively correlated with the expression of the nPR and CK19, a breast epithelial cell marker.

Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction

Nuclear versus Rapid Actions of Steroids

The mechanism of steroid hormone action has been an area of intense study for the last century as steroids are necessary for proper reproductive function. The discovery of the nuclear estrogen receptor in the 1960's and the subsequent discovery of a family of nuclear steroid receptors provided a mechanism by which steroids exerted their effects on target tissues (Evans, 1988). When activated by steroids, the nuclear steroid receptor dimerizes and translocates from the cytosol to the nucleus of the cell where it acts as a transcription factor and alters gene transcription and translation. The process of gene transcription and translation occurs on the order of hours to days, yet there have been reports of rapid steroid actions that occur on the order of seconds to minutes in the absence of gene translation or transcription (Revelii et al., 1998; Wehling, 1997).

Mediators of Rapid Progestin Actions

Nuclear Progestin Receptor (nPR)

Several mammalian studies show that the nPR can initiate rapid, nongenomic signaling both from the cytoplasm and the cell membrane. Studies in breast cancer cells demonstrate that both the ligand activated and unliganded nPR can initiate and respond to growth factor signaling pathways. nPR activation of mitogen activated protein kinases (MAP kinases) is initiated by an interaction between a proline-rich motif on the nPR and SH3 domains on the upstream kinase c-Src (Boonyaratanakornkit et al., 2001). Several

studies also suggest that progesterone acts via this interaction with growth factor signaling pathways to regulate breast cancer cell proliferation (Boonyaratanakornkit et al., 2007; Carnevale et al., 2007; Faivre et al., 2005; Skildum et al., 2005). Other studies suggest that the nPR interacts with the nuclear estrogen receptor (ER) to indirectly activate MAP kinase (Migliaccio et al., 1998). Two domains of the nPR were identified that interacted with the ER and were followed by ER activation of c-Src and MAP kinase (Ballare et al., 2003). The interaction between nPR and ER appears to be involved in progestin induced proliferation of endometrial cells (Vallejo et al., 2005). In addition to nPR activating rapid nongenomic signaling pathways from the cytoplasm, the nPR also appears to contain a highly conserved motif in the ligand binding domain which induces membrane localization (Pedram et al., 2007). There are currently no reports of nPRs in teleost cell membranes and to date all membrane-localized progestin binding observed in teleosts displays the characteristics of the membrane progestin receptor.

Membrane Progestin Receptor (mPR)

Progestins cause final oocyte maturation in the absence of gene transcription or translation in several vertebrate models (Maller and Krebs, 1980; Nagahama et al., 1995) and induces an increase in sperm motility after only a few seconds exposure (Baldi et al., 1998; Thomas and Doughty, 2004). The process of steroid induced final oocyte maturation, the re-entry of an oocyte into meiosis and the production of a fertilizable egg, is being studied in several animal models including fish and amphibians. Studies on oocyte maturation in teleost fishes identified a progestin binding moiety located on ovarian plasma membranes (Patino and Thomas, 1990). This membrane binding-moiety had a high affinity (K_D = 1.5 nM) for the endogenous spotted seatrout (*Cynoscion*

nebulosus) oocyte maturation inducing steroid, 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S);(Thomas and Trant, 1989; Trant and Thomas, 1989). The membrane-localized progestin receptor bound 20β-S with higher affinity than 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) which is different from the steroid specificity profile of the cytosolic nuclear progestin receptor (nPR) in this species (Pinter and Thomas, 1997; Pinter and Thomas, 1999), indicating that the membrane and cytosolic proteins are unrelated. Cloning, sequencing and characterization of the membrane-localized progestin binding protein revealed a novel, seven transmembrane protein and confirmed that the membrane-localized progestin binding protein was unrelated to the nuclear steroid receptor family of proteins (Zhu et al., 2003b). The membrane progestin receptor (mPRα) was capable of binding progestins with a similar affinity (K_D = 30 nM) and steroid specificity pattern to the membrane localized progestin binding protein in the spotted seatrout ovary when expressed in *E. coli*, confirming that the mPRα is the membrane localized progestin receptor in the spotted seatrout (Zhu et al., 2003b). Multiple isoforms of the mPR (mPRα, mPRβ, and mPRγ) have been identified in fish as well as homologues in amphibians and humans (Zhu et al., 2003a). Recent studies suggest that the mPRs are members of the progestin and adiponectin Q receptor (PAQR) family of proteins (Lyons et al., 2004; Tang et al., 2005) which are derived from a different bacterial origin than GPCRs (Thomas et al., 2007). Studies on oocyte maturation in the spotted seatrout, the seatrout mPRα expressed by immortalized human cell lines, the human mPRα over-expressed in immortalized human cell lines and the human mPRs in myometrial cells indicate that the mPRs rapidly activate a pertussis toxin sensitive G protein (Karteris et al., 2007; Pace and Thomas, 2005a; Thomas et al., 2007). Additional studies on final

oocyte maturation in teleosts and expression of the seatrout and zebrafish mPRs in immortalized cell lines demonstrate the activation of p42/44 mitogen-activated protein kinase (MAP kinase) (Hanna et al., 2006; Zhu et al., 2003b) and suggest mPR activation of AKT (Pace and Thomas, 2005a; Pace and Thomas, 2005b). Studies on progestin induced sperm hypermotility in the teleost model indicate the activation of the mPR by progestins and signaling through G proteins, yet the mechanism of mPR signaling in these cells differs from mPR signaling in the oocyte in that the mPR appears to associate with a stimulatory G protein to increase cAMP in sperm (Tubbs, 2007). It is unknown whether the association of the mPR with stimulatory G proteins is isolated to male germ cells or if the mPR associates with multiple G proteins in females.

Progesterone Membrane Binding Component 1 (PGRMC1)

PGRMC1, a putative membrane progestin-binding protein, has been discovered by several different laboratories in several different areas of research under several different names. Other names for PGRMC1 include 25-Dx (Selmin et al., 1996), Hpr6 (Gerdes et al., 1998), VemaA (Runko et al., 1999), ratp28 (Nolte et al., 2000), and IZA (Raza et al., 2001). The connection between PGRMC1 and progesterone was first described in sperm membranes and porcine liver microsomes (Falkenstein et al., 2001; Falkenstein et al., 1999; Falkenstein et al., 1998; Meyer et al., 1998; Meyer et al., 1996). These studies suggest that PGRMC1 is the progesterone binding protein found in porcine liver microsomes and that it binds progesterone with low affinity (K_D = 286 nM). Studies in rat granulosa cells also suggest PGRMC1 binds progesterone with low affinity (K_D = 360 nM). In addition to binding progesterone, PGRMC1 is also suggested to bind heme, cholesterol metabolites and glucocorticoids (reviewed in Cahill, 2007). A homologue of

PGRMC1 has also been cloned in zebrafish and is expressed in the zebrafish ovary (Harris, 2007).

Progestin Action in the Ovarian Follicle

Recent studies have provided much information on the actions and mechanisms of rapid progestin actions involving the mPR on both male and female gametes. However, progestins also have actions in the somatic cells of female reproductive tissues. Progestins increase the steroidogenic capacity of the ovarian follicle by upregulation of steroidogenic enzymes (reviewed in Drummond, 2006). They also initiate ovulation in both teleosts (Pinter and Thomas, 1997; Pinter and Thomas, 1999) and mammals (reviewed in Drummond, 2006). Several studies demonstrate progestin influence on cell cycle control and apoptosis of granulosa cells during the periovulatory period in birds and placental mammals (Chaffin and Stouffer, 2000; Engmann et al., 2006; Mussche and D'Herde, 2001; Peluso et al., 2006; Quirk et al., 2004; Rueda et al., 2000; Svensson et al., 2000; Telleria et al., 1999). Some of the apoptotic actions of progestins in the ovarian follicle are attributable to initiation by the nuclear receptor, yet others occur in the absence of nPR expression. It is unknown if the apoptotic actions of progestins on ovarian follicular cells is conserved in teleosts as there is currently no information on the actions of progestins on teleost ovarian follicular cell cycle control or apoptosis.

Progesterone and Breast Cancer

Progestins also play important roles in normal breast development and in the development and progression of breast cancer in mammals. The nuclear progesterone

receptors are critical for proper lobuloaveolar development though they do not appear to be involved in ductal morphogenesis of the breast (Shyamala, 1999; Shyamala et al., 2000). Progesterone has also been implicated in the development, progression and metastasis of breast cancer (reviewed in Lange, 2007). Of particular interest is that progestin is implicated in the inhibition of breast cancer apoptosis in response to serum starvation and radiation in several cell lines including those which do not express the nuclear progesterone receptor (Moore et al., 2000; Moore et al., 2006; Vares et al., 2004). While the mediator of this progestin action is unknown, our previous studies using nuclear PR negative immortalized human breast cancer cells as models for teleost mPR expression and signaling found that the cells expressed human mPRs. The role of the mPRs in these cells is unknown though they are potential mediators of progestin inhibition of cell death. The expression of the mPRs in human breast cancer cell lines raises the question of whether they are involved in mPR expression in breast cancer development and progression and how their expression relates to other steroid hormone receptors. There is currently no data concerning mPR expression in normal or malignant human breast tissue.

Overall Goals

The overall goals of this research were to test the hypotheses that the mechanism of mPR signaling and the biological function of the mPRs in the somatic cells of female reproductive tissues are conserved across vertebrates and that the mPRs may have significance in human disease. Three specific objectives were developed to examine these hypotheses. The first objective was to examine mPR expression patterns and

signaling in the follicular cells of a teleost fish, the Atlantic croaker *Micropogonias undulatus*, and to examine mPR-mediated progestin inhibition of serum starvation-induced death in these cells. The second objective was to examine mPR expression and signaling in two immortalized human breast cancer cell lines and to examine mPR mediated progesterone inhibition of serum starvation-induced death. The final objective was to examine mPR expression patterns in relation to other steroid receptors in paired normal and malignant human breast biopsies to determine their potential role in breast cancer biology.

Literature Cited

Baldi, E., Luconi, M., Bonaccorsi, L., and Forti, G. (1998). Nongenomic effects of progesterone on spermatozoa: mechanisms of signal transduction and clinical implications. Front Biosci *3*, D1051-1059.

Ballare, C., Uhrig, M., Bechtold, T., Sancho, E., Di Domenico, M., Migliaccio, A., Auricchio, F., and Beato, M. (2003). Two domains of the progesterone receptor interact with the estrogen receptor and are required for progesterone activation of the c-Src/Erk pathway in mammalian cells. Mol Cell Biol *23*, 1994-2008.

Boonyaratanakornkit, V., McGowan, E., Sherman, L., Mancini, M. A., Cheskis, B. J., and Edwards, D. P. (2007). The role of extranuclear signaling actions of progesterone receptor in mediating progesterone regulation of gene expression and the cell cycle. Mol Endocrinol *21*, 359-375.

Boonyaratanakornkit, V., Scott, M. P., Ribon, V., Sherman, L., Andersson, M., Maller, J. L., Miller, W. T., and Edwards, D. P. (2001). Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. Mol Cell *8*, 269-280.

Cahill, M. A. (2007). Progesterone receptor membrane component 1: an integrative review. J Steroid Biochem and Mol Bio *105*, 16-36.

Carnevale, R. P., Proietti, C. J., Salatino, M., Urtreger, A., Peluffo, G., Edwards, D. P., Boonyaratanakornkit, V., Charreau, E. H., Bal de Kier Joffe, E., Schillaci, R., and Elizalde, P. V. (2007). Progestin effects on breast cancer cell proliferation, proteases activation, and in vivo development of metastatic phenotype all depend

on progesterone receptor capacity to activate cytoplasmic signaling pathways. Mol Endocrinol *21*, 1335-1358.

Chaffin, C. L., and Stouffer, R. L. (2000). Role of gonadotrophins and progesterone in the regulation of morphological remodeling and atresia in the monkey peri-ovulatory follicle. Hum Reprod *15*, 2489-2495.

Drummond, A. E. (2006). The role of steroids in follicular growth. Reprod Biol Endocrinol *4*, 16.

Engmann, L., Losel, R., Wehling, M., and Peluso, J. J. (2006). Progesterone regulation of human granulosa/luteal cell viability by an RU486-independent mechanism. J Clin Endocrinol Metab *91*, 4962-4968.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. Science *240*, 889-895.

Faivre, E., Skildum, A., Pierson-Mullany, L., and Lange, C. (2005). Integration of progesterone receptor mediated rapid signaling and nuclear actions in breast cancer cell models: Role of mitogen-activated protein kinases and cell cycle regulators. Steroids *70*, 418-426.

Falkenstein, E., Eisen, C., Schmieding, K., Krautkramer, M., Stein, C., Losel, R., and Wehling, M. (2001). Chemical modification and structural analysis of the progesterone membrane binding protein from porcine liver membranes. Mol Cell Biochem *218*, 71-79.

Falkenstein, E., Heck, M., Gerdes, D., Grube, D., Christ, M., Weigel, M., Buddhikot, M., Meizel, S., and Wehling, M. (1999). Specific progesterone binding to a membrane

protein and related nongenomic effects on Ca2+-fluxes in sperm. Endocrinology *140*, 5999-6002.

Falkenstein, E., Schmieding, K., Lange, A., Meyer, C., Gerdes, D., Welsch, U., and Wehling, M. (1998). Localization of a putative progesterone membrane binding protein in porcine hepatocytes. Cell Mol Biol (Noisy-le-grand) *44*, 571-578.

Gerdes, D., Wehling, M., Leube, B., and Falkenstein, E. (1998). Cloning and tissue expression of two putative steroid membrane receptors. Biol Chem *379*, 907-911.

Hanna, R., Pang, Y., Thomas, P., and Zhu, Y. (2006). Cell-surface expression, progestin binding, and rapid nongenomic signaling of zebrafish membrane progestin receptors α and β in transfected cells. J Endo *190*, 247-260.

Harris, C. (2007) Examination of the role of different classes of progesterone receptors in oocyte maturation in zebrafish, *Danio rerio*, Masters of Science, University of Texas, Port Aransas.

Karteris, E., Zervou, S., Pang, Y., Dong, J., Hillhouse, E., Randeva, H., and Thomas, P. (2007). Progesterone signaling in human myometrium through two novel membrane G protein coupled receptors: Potential role in functional progesterone withdrawal at term. Mol Endocrinol *20*, 1519-1534.

Lange, C. A. (2007). Challenges to defining a role for progesterone in breast cancer. Steroids.

Lyons, T. J., Villa, N. Y., Regalla, L. M., Kupchak, B. R., Vagstad, A., and Eide, D. J. (2004). Metalloregulation of yeast membrane steroid receptor homologs. Proc Natl Acad Sci U S A *101*, 5506-5511.

Maller, J. L., and Krebs, E. G. (1980). Regulation of oocyte maturation. Curr Top Cell Regul *16*, 271-311.

Meyer, C., Schmid, R., Schmieding, K., Falkenstein, E., and Wehling, M. (1998). Characterization of high affinity progesterone-binding membrane proteins by anti-peptide antiserum. Steroids *63*, 111-116.

Meyer, C., Schmid, R., Scriba, P. C., and Wehling, M. (1996). Purification and partial sequencing of high-affinity progesterone-binding site(s) from porcine liver membranes. Eur J Biochem *239*, 726-731.

Migliaccio, A., Piccolo, D., Castoria, G., Di Domenico, M., Bilancio, A., Lombardi, M., Gong, W., Beato, M., and Auricchio, F. (1998). Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. Embo J *17*, 2008-2018.

Moore, M. R., Conover, J. L., and Franks, K. M. (2000). Progestin effects on long-term growth, death, and Bcl-xL in breast cancer cells. Biochem Biophys Res Commun *277*, 650-654.

Moore, M. R., Spence, J. B., Kiningham, K. K., and Dillon, J. L. (2006). Progestin inhibition of cell death in human breast cancer cell lines. J Steroid Biochem Mol Biol *98*, 218-227.

Mussche, S., and D'Herde, K. (2001). Contribution of progesterone, follicle stimulating hormone and glucocorticoids in survival of serum-free cultured granulosa cell explants. J Endo *169*, 321-331.

Nagahama, Y., Yoshikuni, M., Yamashita, M., Tokumoto, T., and Katsu, Y. (1995). Regulation of oocyte growth and maturation in fish. Curr Top Cell Regul *30*, 103-145.

Nolte, I., Jeckel, D., Wieland, F. T., and Sohn, K. (2000). Localization and topology of ratp28, a member of a novel family of putative steroid-binding proteins. Biochim Biophys Acta *1543*, 123-130.

Pace, M. C., and Thomas, P. (2005a). Activation of a pertussis toxin-sensitive, inhibitory G-protein is necessary for steroid-mediated oocyte maturation in spotted seatrout. Dev Biol *285*, 70-79.

Pace, M. C., and Thomas, P. (2005b). Steroid-induced oocyte maturation in Atlantic croaker (Micropogonias undulatus) is dependent on activation of the phosphatidylinositol 3-kinase/Akt signal transduction pathway. Biol Reprod *73*, 988-996.

Patino, P., and Thomas, P. (1990). Characterization of membrane receptor activity for 17α,20β,21-trihydroxy-4-pregnen-3-one in ovaries of spotted seatrout (*Cynoscion nebulosus*). Gen Comp Endo *78*, 204-217.

Pedram, A., Razandi, M., Sainson, R. C. A., Kim, J. K., Hughes, C. C., and Levin, E. R. (2007). A conserved mechanism of steroid receptor translocation to the plasma membrane. J Biol Chem *282*, 22278-22288.

Peluso, J., Pappalardo, A., Losel, R., and Wehling, M. (2006). Progesterone membrane receptor component 1 expression in the immature rat ovary and its role in mediating progesterone's antiapoptotic action. Endocrinology *147*, 3133-3140.

Pinter, J., and Thomas, P. (1997). The ovarian progestogen receptor in the spotted seatrout, Cynoscion nebulosus, demonstrates steroid specificity different from progesterone receptors in other vertebrates. J Steroid Biochem Mol Biol *60*, 113-119.

Pinter, J., and Thomas, P. (1999). Induction of ovulation of mature oocytes by the maturation-inducing steroid 17,20beta,21-trihydroxy-4-pregnen-3-one in the spotted seatrout. Gen Comp Endocrinol *115*, 200-209.

Quirk, S., Cowan, R., and Harman, R. (2004). Progesterone receptor and the cell cycle modulate apoptosis in granulosa cells. Endocrinology *145*, 5033-2043.

Raza, F. S., Takemori, H., Tojo, H., Okamoto, M., and Vinson, G. P. (2001). Identification of the rat adrenal zona fasciculata/reticularis specific protein, inner zone antigen (IZAg), as the putative membrane progesterone receptor. Eur J Biochem *268*, 2141-2147.

Revelii, A., Massobria, M., and Tesarik, J. (1998). Nongenomic actions of steroid hormones in reproductive tissues. Endocr Rev *19*, 3-17.

Rueda, B., Hendry, I., Hendry, W., Stormshak, F., Slayden, O., and Davis, J. (2000). Decreased progesterone levels and progesterone receptor antagonists promote apoptotic cell death in bovine luteal cells. Bio Rep *62*, 269-276.

Runko, E., Wideman, C., and Kaprielian, Z. (1999). Cloning and expression of VEMA: a novel ventral midline antigen in the rat CNS. Mol Cell Neurosci *14*, 428-443.

Selmin, O., Lucier, G. W., Clark, G. C., Tritscher, A. M., Vanden Heuvel, J. P., Gastel, J. A., Walker, N. J., Sutter, T. R., and Bell, D. A. (1996). Isolation and

characterization of a novel gene induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin in rat liver. Carcinogenesis *17*, 2609-2615.

Shyamala, G. (1999). Progesterone signaling and mammary gland morphogenesis. J Mammary Gland Biol *4*, 89-104.

Shyamala, G., Yang, X., Cardiff, R. D., and Dale, E. (2000). Impact of progesterone receptor on cell-fate decisions during mammary gland development. Proc Natl Acad Sci U S A *97*, 3044-3049.

Skildum, A., Faivre, E., and Lange, C. (2005). Progesterone receptors induce cell cycle progression via activation of mitogen-activated protein kinases. Mol Endo *19*, 327-339.

Svensson, E. C., Markstrom, E., Andersson, M., and Billig, H. (2000). Progesterone receptor-mediated inhibition of apoptosis in granulosa cells isolated from rats treated with human chorionic gonadotropin. Biol Reprod *63*, 1457-1464.

Tang, Y. T., Hu, T., Arterburn, M., Boyle, B., Bright, J. M., Emtage, P. C., and Funk, W. D. (2005). PAQR proteins: a novel membrane receptor family defined by an ancient 7-transmembrane pass motif. J Mol Evol *61*, 372-380.

Telleria, C. M., Stocco, C. O., Stati, A. O., and Deis, R. P. (1999). Progesterone receptor is not required for progesterone action in the rat corpus luteum of pregnancy. Steroids *64*, 760-766.

Thomas, P., and Doughty, K. (2004). Disruption of rapid, nongenomic steroid actions by environmental chemicals: interference with progestin stimulation of sperm motility in Atlantic croaker. Environ Sci Technol *38*, 6328-6332.

Thomas, P., Pang, Y., Dong, J., Groenen, P., Kelder, J., de Vlieg, J., Zhu, Y., and Tubbs, C. (2007). Steroid and G protein binding characteristics of the seatrout and human progestin membrane receptor alpha subtypes and their evolutionary origins. Endocrinology *148*, 705-718.

Thomas, P., and Trant, J. (1989). Evidence that 17α,20β,21-trihydroxy-4-pregnen-3-one is a maturation-inducing steroid in spotted seatrout. Fish Phys Biochem *7*, 185-191.

Trant, J., and Thomas, P. (1989). Isolation of a novel maturation-inducing steroid produced *in vitro* by ovaries of Atlantic croaker. Gen Comp Endo *75*, 397-404.

Tubbs, C. (2007) Mechanisms of Progestin–Stimulated Sperm Hypermotility in Two Teleosts: the Atlantic Croaker (Micropogonias undulatus) and the Southern Flounder (*Platylicthys lethigstomata*), Doctoral Dissertation, University of Texas, Port Aransas.

Vallejo, G., Ballare, C., Baranao, J. L., Beato, M., and Saragueta, P. (2005). Progestin activation of nongenomic pathways via cross talk of progesterone receptor with estrogen receptor beta induces proliferation of endometrial stromal cells. Mol Endocrinol *19*, 3023-3037.

Vares, G., Ory, K., Lectard, B., Levalois, C., Altmeyer-Morel, S., Chevillard, S., and Lebeau, J. (2004). Progesterone prevents radiation-induced apoptosis in breast cancer cells. Oncogene *23*, 4603-4613.

Wehling, M. (1997). Specific, nongenomic actions of steroid hormones. Annu Rev Physiol *59*, 365-393.

Zhu, Y., Bond, J., and Thomas, P. (2003a). Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. Proc Natl Acad Sci U S A *100*, 2237-2242.

Zhu, Y., Rice, C. D., Pang, Y., Pace, M., and Thomas, P. (2003b). Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc Natl Acad Sci U S A *100*, 2231-2236.

Chapter 2

Progestin signaling through mPRα in Atlantic croaker granulosa/theca cell co-cultures: possible involvement in progestin inhibition of apoptosis

Summary

Although there is substantial evidence that membrane progestin receptors (mPRs) perform a critical physiological role in meiotic maturation of fish oocytes, it is not known whether they are also intermediaries in progestin signaling in the surrounding follicular cells. Therefore, expression of mPRα, its signaling characteristics, and its physiological functions were investigated in co-cultured granulosa and theca cells (G/T cells) isolated from Atlantic croaker (*Micropogonias undulatus*) ovaries. Here we show that the mPRα protein is expressed on the plasma membranes of both granulosa and theca cells and is associated with the presence of a single high affinity, limited capacity, specific progestin (17,20β,21-trihydroxy-4-pregnen-3-one, 20β-S) binding site on G/T cell membranes with the characteristics of mPRα. Treatment of the ovarian follicle cells with 20β-S caused rapid G protein activation, a transient decrease in cAMP levels and activation of MAP kinase, whereas the nuclear progestin receptor (nPR) agonist, R5020, was ineffective. Pertussis toxin treatment blocked the 20β-S–induced decrease in cAMP levels and decreased 20β-S binding to the membrane receptor, both of which suggest the progestin receptor is coupled to an inhibitory G protein (G_i), consistent with its identity as mPRα. In addition, co-immunoprecipitation experiments confirmed that mPRα is

coupled to an inhibitory G protein in croaker G/T cells. A potential physiological role of mPRα in mediating the anti-apoptotic actions of progestins was examined in cultured croaker G/T cells. The results showed that 20β-S and other progestins which activate mPRα decrease cell death due to serum starvation, whereas R5020, which does not activate mPRα, was inactive. This is the first study to demonstrate progestin inhibition of G/T cell death in teleosts. The finding that this anti-apoptotic action is mediated by specific mPRα agonists suggests it is mediated through this receptor and may indicate a novel function for mPRα in vertebrate ovarian follicular cells.

Introduction

Rapid, nongenomic actions of progestins have been known for several decades and have been shown to have an important role in initiating the process of final oocyte maturation in fish and amphibians. Until recently the identity of the receptor mediating these rapid nongenomic actions of progestins on oocytes was unknown. A membrane progestin receptor that is the likely intermediary in progestin induction of oocyte maturation in fish was first biochemically characterized in the ovaries of spotted seatrout, *Cynoscion nebulosus* (Patiño and Thomas, 1990). The receptor protein has high affinity and limited capacity specific binding for the endogenous progestin 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) (K_D = 1.5 nM, B_{max} = 7.7 nmol/gram ovary) (Patiño and Thomas, 1990; Thomas and Das, 1997) with ligand binding kinetics and a steroid specificity profile that are very different from those of the nuclear progestin receptor characterized in the same species (Patiño and Thomas, 1990; Pinter and Thomas, 1995; Pinter and Thomas, 1997), suggesting it is a novel receptor protein however, until

recently, the identity of this receptor was unknown. A novel cDNA, unrelated to nuclear steroid receptors or any other hormone receptors, was discovered in a spotted seatrout ovarian library that has the characteristics of the membrane progestin receptor mediating progestin induction of oocyte maturation in the species (Zhu et al., 2003b). The cDNA encodes a ~ 40 kDa protein, named membrane progestin receptor alpha (mPRα), that has seven transmembrane domains and specifically binds 20β-S with high affinity in both recombinant *E. coli* expression systems (K_D = 30 nM) (Zhu et al., 2003b) and in mammalian expression systems (K_D = 7.58 nM) (Thomas et al., 2007). Additional studies on mPRα suggested that it is capable of activating pertussis toxin-sensitive inhibitory G proteins in mammalian expression systems (Thomas et al., 2007). Multiple isoforms of the mPR have been cloned in several vertebrate species including humans (Zhu et al., 2003a), and recent phylogenetic analyses suggest that they are members of the progestin and adiponectin Q receptor (PAQR) family of proteins (Lyons et al., 2004; Tang et al., 2005). mPR protein expression on the oocyte cell membrane has been demonstrated in several models including spotted seatrout (Pace and Thomas, 2005a; Zhu et al., 2003b) zebrafish (mPRα (Hanna et al., 2006)), the goldfish (mPRα (Tokumoto et al., 2006)) and *Xenopus* (mPRβ (Josefsberg Ben-Yehoshua et al., 2007) as well mRNA expression in the ovary of the catfish (Kazeto et al., 2005), sheep (Ashley et al., 2006) and human (Zhu et al., 2003a). Of particular interest are reports of mPRα mRNA expression in the corpus luteum of rat (Cai and Stocco, 2005) and sheep (Ashley et al., 2006) suggesting that mPRα is expressed by ovarian endocrine cells as well as on the oocyte. There is currently no evidence that mPRs are expressed in follicle cells. However, recent reports of rapid, non-genomic actions of progestins in granulosa/luteal

cells from a variety of species (Chaffin and Stouffer, 2000; Engmann et al., 2006; Peluso et al., 2006; Telleria et al., 1999) suggest membrane progestin receptors are present in these cells.

Progesterone treatment has been shown to increase granulosa cell survival in .oviparous birds (quail (Mussche and D'Herde, 2001)) as well placental mammals (cow (Quirk et al., 2004; Rueda et al., 2000), rat (Peluso et al., 2006; Svensson et al., 2000; Telleria et al., 1999), primate (Chaffin and Stouffer, 2000) and human (Engmann et al., 2006)). The mechanism of progesterone's anti-apoptotic action remains unclear because, although it has been attributed to the presence of nPR in some cell models (Mussche and D'Herde, 2001; Quirk et al., 2004; Rueda et al., 2000; Svensson et al., 2000), it has also been demonstrated in cells that lack nPR activity (Chaffin and Stouffer, 2000; Engmann et al., 2006; Peluso et al., 2006; Telleria et al., 1999). Another potential mediator of progesterone's anti-apoptotic actions, mPR, has not been investigated to date. The sciaenid fish *Micropogonias undulatus*, the Atlantic croaker, is a well characterized teleost model used to study the hypothalamic- pituitary- gonadal reproductive axis, ovarian steroidogenesis and oocyte maturation. The croaker oocyte is surrounded by monolayers of granulosa and theca cells which can be co-cultured making it an excellent model for examining the actions of progestins on teleost follicular cells independent of the oocyte (Benninghoff and Thomas, 2006).

A major objective of this study was to examine whether mPRα is expressed in granulosa and theca cells of the Atlantic croaker and to determine the signal transduction pathways activated by progestins through the receptor. Another objective was to determine the role of progestins in serum starvation-induced granulosa cell death in this

teleost fish model in order to examine the conservation of this progestin action across vertebrates. A final goal was to obtain preliminary information on the likely identity of the progestin receptor that mediates these actions.

Materials and Methods

Chemicals

Chemicals were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted. 20β-S, 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P), estradiol-17β, testosterone and cortisol were purchased from Steraloids (Newport, RI). R5020 was purchased from PerkinElmer (Waltham, MA). Organon 02-0 and Organon 13-0 were obtained from Organon (Roseland, NJ). Radiolabled 20β-S and 17,20β-P precursors were purchased from American Radiolabeled Chemicals (St. Louis, MO) and PerkinElmer NEN (Waltham, MA) respectively. [1,2, ^{3}H] 11-deoxycortisol and [1,2,6,7 ^{3}H] 17α hydroxyprogesterone were converted into [1,2, ^{3}H] 17α,20β,21-trihydroxy-4-pregnen-3-one and [1,2,6,7 ^{3}H] 17α,20β dihydroxy-4-pregnen-3-one respectively, with 20β-hydroxysteroid dehydrogenase(20β-HSD) as described previously (Scott et al., 1982).

Animal care and cell culture

Adult Atlantic croaker were purchased from local bait shops near Port Aransas, Texas, prior to the reproductive season and maintained in 11,000L re-circulating filtered seawater tanks at 24^{o}C with 13L:11D photoperiod at the University of Texas Marine Science Institute. Fish were fed a diet of commercial pellets and shrimp daily.

Croaker were humanely sacrificed according to NIH guidelines by procedures approved by the University of Texas at Austin IUCAC. Primary granulosa and theca cell (G/T) cultures were obtained as described previously (Benninghoff and Thomas, 2006). Briefly, ovaries were harvested from sacrificed fish and mechanically dispersed with repeated serological pipetting. Connective tissue was digested in 0.1% collagenase with gentle rotation for 1.5 hours followed by repeated serological pipetting to shear follicle cells from the oocytes. Most of the oocytes settled to the bottom of the culture tube and the supernatant containing the G/T cells was passed through a 100 μm mesh nitex screen to remove any remaining oocytes. The supernatant was then layered above a 45% percoll pad and centrifuged at 2800 X g for 20min. G/T cells formed a layer at the percoll interface. The cells were collected and washed with Dubecco's Modified Eagle Medium (DMEM) prior to culture at an initial density of $2.5x10^5$ cells/mL in DMEM supplemented with 2 percent bovine calf serum. Cells were harvested the next day for characterization of mPRα expression and progestin signaling

RT-PCR

G/T cells were harvested directly from culture in Tri-Reagent (Sigma Aldrich, St. Louis, MO) and RNA was isolated following the manufacturer's protocols. Isolated RNA was DNase treated to remove any DNA using a DNA free RNA kit (Zymo Research, Orange CA). Reverse transcription was performed on 1μg total RNA using Platinum Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and 500 μg/mL oligo(dT)s. PCR was performed on diluted (1:10) reverse transcription reaction using Platinum PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA) with 200 nM final primer concentration. Primers were designed against croaker mPRα (GenBank

Accession No. EU095257, 5'GGCAGCAGAAGAAATAGGCG, 5' GCTGGCGCTACTACTTTCTCA), croaker nPR (Dr. R. Patiño, personal communication) (5'GGCTCCTTTTCGTCTTTGATG, 5'CCTGATTGAAGCTGGGTACAGT), zebrafish PGRMC1 (GenBank Accession No. BX842703, 5'CGCTGCCCAAACTCAAGA, 5'-GTTTGGGTCCGCTCTAATC) and croaker 18S (5'GTTAATTCCGATAACGAACGAGACTC, 5'ACAGACCTGTTATTGCTCAATCTCGTG) following the manufacturer's instructions with an annealing temperature of 55^{o}C for 35 cycles. Reactions were mixed 1:6 with BlueJuice Gel Loading Buffer (Invitrogen, Carlsbad, CA) and run on a 1% agarose gel and photographed.

W*estern Blot Analyses*

G/T cells were plated in 150cm^2 cell culture dishes and harvested by scraping the following day in ice cold PBS for mPR protein detection. The cells were briefly sonicated (10 seconds at a setting of 2.5 using a 550 Sonic Dismembrator, Fisher Scientific, Hampton, NH) on ice in the presence of HALT protease inhibitor cocktail (Pierce, Rockford, IL). Nuclear fractions and unlysed cells were removed by centrifugation at 500 X g for 7 minutes at 4^{o}C. The resulting supernatant was centrifuged at 20,000 X g for 20 minutes to isolate the plasma membrane fraction. The resulting pellet was resuspended in 1X reducing loading buffer (Pierce, Rockford, IL) and boiled for 10 min. 15 μg membrane protein was loaded and run on a 10% SDS PAGE gel and the protein bands on the gel were transferred to nitrocellulose membranes (Biorad, Hercules, CA). Following transfer, membranes were washed 3 times for 5 minutes with PBS and blocked in 5% nonfat milk and 0.1% Tween-20 in PBS for one hour at room

temperature. Croaker mPRα was detected using an antibody generated against mPRα of the spotted seatrout (*Cynoscion nebulosus*). The seatrout mPRα antibody was produced against the N-terminal peptide sequence YRQPDQSWRYYFLTL. This portion of the mPRα protein is identical in seatrout and croaker (GenBank Accession No. AF262028 and EU095257 respectively). Affinity purified spotted seatrout mPRα rabbit antiserum was added to membranes at a final dilution of 1:5,000 in PBS with 5% nonfat milk and incubated overnight at 4^{o}C. Membranes were washed 3X 5 min with TBS-T (20 mM Tris base, 150 mM NaCl, 0.1% Tween 20, pH 7.6) and incubated with secondary HRP-linked goat anti-rabbit antibody (AbCam, Cambridge, MA) at a final concentration of 1:5000 in TBS-T with 5% nonfat milk for one hour at room temperature. Membranes were again washed 3 times for 5 minutes with TBS-T and SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford IL) was used to visualize mPRα on ECL hyperfilm (Amersham, Piscataway, NJ).

MAP kinase activation assay

G/T cells were cultured in 6-well cell culture plates overnight and were serum starved for 24-48 hours prior to steroid treatment and measurement of ERK activation. The serum free medium was replaced with DMEM containing steroids or vehicle and incubated for 5, 10, 15, 30 or 60 minutes. Upon termination of the incubation, the media was rapidly removed by suction and the cells were harvested directly into 1X reducing loading dye (Pierce, Rockford, IL). Samples were briefly sonicated and boiled for 10 minutes prior to running 20 µL per sample on a 10% SDS PAGE gel and subsequent transfer of the protein bands to PVDF membranes (Biorad, Hercules, CA). Membranes were probed using antibodies directed against total p42/44 (ERK) and phosphor-p42/44

(phosphor-ERK) (Biomol, Plymouth Meeting, PA) following the manufacturer's suggestions and visualized using SuperSignal West Pico chemiluminescent substrate on hyperfilm. Image J (NIH) was used to quantify band density.

Immunocytochemistry

Cultured G/T cells were grown on glass cover slides. All the following steps were conducted at 4^{o}C. Cells were fixed in 2% paraformaldehyde followed by three x five minute washings with PBS. Cells were blocked in 3% BSA in PBS for one hour and then incubated overnight with mPRα primary antibody at a concentration of 1:5000. Negative controls were incubated in the absence of primary antibody. Cells were washed 3 times for five minutes with PBS followed by incubation with Alexaflor-488 linked goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR) at a dilution of 1:5000 in PBS with 3% BSA for 1 hour. Cells were washed 3 times for 10 minutes each and incubated with 300 nM 6-diamidino-2-phenylindole (DAPI) for 5 minutes. Cells were washed in PBS twice for 10 minutes prior to mounting using ProLong Gold Antifade Reagent (Molecular Probes, Eugene, OR) and visualized using a Nikon Eclipse E600 fluorescent microscope or a Nikon C1 confocal microscope.

[^{35}S]-GTPγS binding to G/T cell membranes

G/T cells were isolated from croaker ovaries and used immediately for the [^{35}S] GTPγS binding assay. Following enzyme digestion the isolated follicle cells were centrifuged at 500 X g for 5 minutes to pellet live cells and resuspended in 4mL ice cold HAED (25 mM HEPES, 10 mM NaCl, 1 mM Dithioerythritol, 1 mM EDTA, pH 7.6) with HALT protease inhibitor cocktail (Pierce, Rockford, IL). Cells were sonicated for 10 seconds at a setting of 2.5 and the resulting homogenate was centrifuged at 1000 Xx g

for 7 minutes. The supernatant was then centrifuged for 20 minutes at 20,000 X g to pellet the plasma membranes. The membranes were resuspended in binding buffer (100 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.6 mM EDTA, 0.1% BSA, 50 mM Tris-HCl, pH 7.6) with HALT protease inhibitor cocktail to a concentration of 0.5 mg/mL protein. Membranes were pre-incubated with 10 μM GDP and 0.5 nM [^{35}S] GTPγ-S (Amersham, Piscataway, NJ) in the presence or absence of 500 nM unlabeled GTPγ-S at 24^{o}C for 5 minutes followed by incubation in the presence or absence of various concentrations of steroids for 20 minutes with gentle agitation. The reaction was terminated by rapid filtration onto Whatmann GF/B filters presoaked in binding buffer lacking BSA and washed twice with 4 mL binding buffer lacking BSA. Specific binding was determined by subtracting nonspecific from total binding.

Progestin Receptor Binding Assay

The binding assay was conducted following procedures described previously (Patino and Thomas, 1990). Cells were harvested and membranes isolated as described above with the final membrane pellet being resuspended in HAED. For saturation binding analysis, membranes were incubated with increasing concentrations (0.25-11 nM) of [^{3}H]20β-S at 4^{o}C for 30 minutes. Nonspecific binding was determined in the presence of 1000 fold excess unlabeled 20β-S. The incubation was terminated by rapid filtration onto Whatmann GF/B filters presoaked in HAED minus dithioerythritol (HAEW) using a cell harvester (Brandel, Gaithersburg MD). The [^{3}H]20β-S bound to the membranes was measured in a scintillation counter (Beckmann LS6000SC) and saturation curves were generated using GraphPad software (San Diego, CA). Specific binding was determined by subtracting the nonspecific binding from the total binding.

Competition studies were conducted using a similar protocol. Membranes were incubated in the presence of 100 nM competitor or ethanol vehicle with 2 nM [^{3}H]20β-S for 30 minutes at 4^{o}C. The reaction was terminated as described previously. The influence of pertussis toxin (PTX) (List Biological Campbell, CA) on [^{3}H]20β-S binding was determined by pre-incubating G/T cells with 1-6 μg activated PTX or heat-inactivated PTX (Kaslow et al., 1987) per milliliter media at 18^{o}C for 20 minutes prior to membrane isolation. Membranes were incubated with 2 nM [^{3}H]20β-S at 4^{o}C for 30 minutes. Nonspecific binding was determined in the presence of 1000 fold excess unlabeled 20β-S.

Competitive binding to the nuclear PR was conducted as described previously (Pinter and Thomas, 1995). Briefly, cytosolic fractions were isolated from croaker ovarian fragments in TEDM buffer (10 mM Tris, 1 mM EDTA, 5 mM dithiothreitol, 10 mM sodium molybdate, pH 7.4) and incubated with 5 nM [^{3}H]17,20B-P in the presence or absence of 500 nM 17,20β-P and various concentrations of competitor for 12-24 hours at 4^{o}C. Incubations were terminated by adding 500 μL ice-cold dextran-coated charcoal solution (0.1% dextran, 0.4% activated charcoal in TEDM) and incubating the mixture for 5 minutes at 4^{o}C followed by and centrifugation at 5000 X g for 5 minutes.

Co-Immunoprecipitation of G protein α_i subunit with mPRα

Cultured G/T cells from 5 150cm^2 culture plates were harvested in ice-cold HAED and lysed by brief sonication on ice. Nuclear fractions were pelleted by centrifugation at 1000 X g for 7 minutes. The supernatant was centrifuged at 20,000 X g for 30 minutes to pellet plasma membranes. Plasma membranes were resuspended in binding buffer with 100 μM GTP and 10 μM GDP to a concentration of 2 mg/mL protein

and 200 μL samples incubated in the presence or absence of 20β-S for 45 minutes at 4°C. Membranes were pelleted with centrifugation and resuspended in 500 μL solubilization buffer (150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Triton X-100, pH 7.4) and incubated at 4°C with rotation. Samples were centrifuged to remove any unsolubilized membrane. Antibody directed against $G\alpha_{i,o,t,z}$ (Santa Cruz, Santa Cruz CA) was added at a dilution of 1:200 and samples were incubated with rotation for two hours before the addition 25 μL of AgG coated beads (Santa Cruz, Santa Cruz CA) and further incubation overnight. Beads were pelleted with centrifugation and washed three times with PBS. Beads were resuspended in 1X reducing western loading dye (Pierce, Rockford, IL) and boiled for 10 minutes. Samples were Western blotted for mPRα.

Cyclic AMP assay

Cells were cultured in 6-well cell culture dishes and then serum starved for 36 hours. Cells were washed twice with PBS and pretreated with serum free medium containing 10 μM IBMX dissolved in DMSO for 20-30 minutes. The pretreatment medium was replaced with media containing both IBMX and 100 nM 20β-S or vehicle and the mixture was incubated for various periods ranging from 1 to 30 minutes. At the end of the exposure period, media were quickly removed and 100 μL 0.1M HCl was added to each well. After 20 minutes of incubation with 0.1 M HCl at room temperature the cells were scraped from the plates, collected and stored at -20°C until assayed in duplicate for cAMP using an EIA kit according to the manufacturer's instructions for cAMP measurement with acetylation (Cayman Chemical, Ann Arbor, MI). For PTX experiments cells were pre-incubated for 30 minutes with 2 μM forskolin in the presence or absence of 0.5 μg/mL activated PTX or heat-inactivated PTX (hiPTX) (Kaslow et al.,

1987)(List Biological, Campbell, CA). 100 nM 20β-S or ethanol (final ethanol concentration did not exceed 0.1% media volume) was added after the pre-incubation period and the mixture was incubated for an additional 20 minutes. At the end of the incubation period the media was rapidly removed and the cell extracts were processed for cAMP measurement as described previously.

Serum starvation- induced cell death

Cell death assays protocols were adapted from those described for breast cancer cells (Moore et al., 2006). G/T cells were cultured in 25cm^2 filter-top flasks in a humidified 24^{o}C chamber overnight. The cells were washed twice with PBS (136 mM NaCl, 2.7 mM KCl, 12.2 mM $NaPO_4$, 1.7 mM KH_2PO_4, pH 7.4) to remove all traces of serum and triplicate flasks were cultured in serum free DMEM in the presence of ethanol vehicle alone (ethanol concentration did not exceed 0.1% total volume), or various steroids (1-100nM) dissolved in the same volume of ethanol. The cells were incubated for 5-8 days without media change with additional steroid added every two days. At the end of the experiment the media were removed and adherent cells were harvested in Hank's saline (5.36 mM KCl, 0.4 mM KH_2PO_4, 4.1 mM $NaHCO_3$, 137 mM NaCl, 0.33 mM Na_2HPO_4, 5.5 mM D-Glucose pH 7.4). The combined media and adherent cells were centrifuged at 1000 X g for 3 minutes to pellet the cells. The supernatant was carefully poured off and the pellet was gently resuspended in 250-500 µL Hank's saline. The dead cells were identified under a microscope using trypan blue exclusion (Freshney, 1994). 100 µL 0.4% filtered Trypan Blue Stain was added and the cells were incubated for 5 minutes. Cells were loaded onto a hemocytometer and viability was determined by Trypan Blue Stain exclusion. A total of 500 cells from each flask were scored for

viability. This experiment was performed three times. In other experiments cells were stained for DNA fragmentation (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling, TUNEL) using the ApoAlert DNA Fragmentation Assay Kit (Clontech, Mountain View CA) as per manufacturer's instructions. TUNEL labeled cells were counted for five random fields of view on a Nikon Eclipse E600 fluorescent microscope. This experiment was performed three times.

Statistical Analyses

One way ANOVA with either Dunnett's multiple comparison or Bonferroni's multiple comparison was used to determine statistical differences between control and experimental treatments using GraphPad Prism (San Diego, CA). Square root or log transformations of the data were used as indicated in order to remove significant differences in variance.

Results

Progestin receptor expression and localization in G/T cell co-cultures

The presence of mPRα mRNA in croaker G/T cell co-cultures was demonstrated by reverse-transcription polymerase chain reaction (RT-PCR) which amplified a product of the predicted size, 609 bp (Figure 2-1A). In addition, the presence of the croaker nuclear PR mRNA in these cells was confirmed by RT-PCR (product 351 bp). Moreover, a product of the expected size (401 bp) was amplified using primers designed against a conserved sequence of the putative progesterone receptor, PGMRC1, in zebrafish (Figure 2-1A). Reverse transcriptase negative controls showed lack of DNA contamination (Figure 2-1A). Immunofluorecent analyses demonstrated that the mPRα protein is

expressed on the plasma membranes of the G/T cells. Western blot analysis of G/T cell membranes using an antibody directed against the N terminus of the seatrout mPRα resulted in the detection of a single immunoreactive band at 80 kDa, likely representing an mPRα dimer (Figure 2-1B). Immunocytochemistry using the same antibody shows specific mPRα localization on the plasma membranes of these cells (Figure 2-2A) and its presence on both granulosa and theca cells in the follicular culture (Figure 2-2B). Intracellular, perinuclear mPRα staining can also be seen in some cells and is likely mPRα in the endoplasmic reticulum. Visualizing the cells under confocal microscopy reveals punctate staining of mPRα on the surface of the follicular cells (Figure 2-2C). Cells stained with only secondary antibody did not demonstrate staining (Figure 2-2D).

[^{3}H]20β-S binding to G/T cell membranes

The progestin hormone in croaker, 20β-S, bound to the plasma membranes of croaker G/T cell co-cultures with characteristics typical of steroid membrane receptors. Saturation binding analysis revealed a single, high affinity (K_D = 1.7 nM 20β-S), limited capacity (B_{max} = 0.0975 nM or 0.39 fmol/mg protein) progestin binding site on follicular plasma membranes (Figure 2-3A). Single point competitive binding studies showed that the binding is specific for progestins. 100 nM 20β-S, progesterone, and the synthetic progestins Organon 02-0 and Organon 13-0, caused significant displacement of [^{3} H]20β-S from the binding site, whereas 100 nM testosterone displayed low affinity for the receptor and estradiol-17β and cortisol were ineffective as competitors (Figure 2-3B). In order to determine whether synthetic progestins may be used to distinguish between the actions initiated by the mPR and the nPR, competition studies were performed with Organon 02-0 (J.Kelder, Organon, personal communication.) which has been shown to

have mPRα agonist activity on progestin induction of oocyte maturation in zebrafish (Harris, 2007), and with the synthetic nuclear PR agonist, R5020, which has low binding affinity for seatrout and human mPRαs in mammalian expression systems (Thomas et al., 2007). Competition for mPR binding sites on croaker ovarian membranes showed that Organon 02-0 (EC50 = 44 nM) bound mPR with an affinity approximately one order of magnitude lower than the endogenous progestin 20β-S (EC50 = 2.6 nM), whereas R5020 (EC50 = 297 nM) bound to the mPR with an affinity two orders of magnitude lower than 20β-S (Figure 2-3C). Competition for nPR binding sites in croaker ovarian cytosolic preparations showed that the binding affinity of R5020 (EC50 = 6.3 nM) for the nuclear receptor was within one order of magnitude of that of the endogenous progestin 17,20β-P (EC50 = 0.73 nM) (Figure 2-3D), but was two orders of magnitude higher than its affinity for the mPR. It is concluded from these competitive binding studies that Organon-02 is a candidate for a mPR agonist in Atlantic croaker and that concentrations of R5020 in the range of 10-100 nM should act as a selective agonist of nPR-mediated progestin actions.

Possible coupling of the membrane progestin receptor in ovarian G/T cells to inhibitory G proteins (G_i and G_o) was investigated by examining specific binding of [^{3}H]20β-S to follicle cell membranes after uncoupling the G proteins by pretreatment with PTX. Pre-incubation of cells with activated PTX significantly decreased specific [^{3}H]20β-S binding by 30 percent compared to control and heat-inactivated PTX determined by one way ANOVA and Dunnett's multiple comparison test $p < 0.05$, $n = 3$ (Figure 2-3E), suggesting that the progestin receptor on G/T cell membranes is coupled to an inhibitory G protein.

G protein activation in response to 20β-S exposure

In order to determine whether the membrane progestin receptor activates G proteins, binding of [^{35}S]GTPγ-S to G/T cell membranes was assessed after treatment with progestins. [^{35}S]GTPγ-S binding to G/T cell membranes increased significantly after treatment with 20-100 nM 20β-S and 20nM Organon 02-0 above that measured in the vehicle (ethanol) treated controls, but was not altered in response to treatment with 20 nM R5020 (one way ANOVA $^{**}p < 0.001$, n = 7; and Dunnett's multiple comparison test $^{*}p < 0.05$, n = 3, Figure 2-4A). These findings are further evidence that the progestin membrane receptor in croaker G/T cells activates G proteins upon ligand binding and are consistent with its identity as mPRα.

Co-immunoprecipitation of mPRα with an inhibitory G protein

mPRα was co-immunoprecipitated using an antibody directed against $G\alpha_{i,o,t,z}$, followed by Western blot analysis using a mPRα antibody (Figure 2-4B), suggesting that mPRα is coupled to an inhibitory G protein. In addition, the amount of mPRα protein co-immunoprecipitated with G_i was decreased after pretreatment with higher concentrations of 20β-S (290 nM, Fig.2-4B, lane1), while the amounts of G_i immunoprecipitated after treatment with 29 nM 20β-S were similar to the no treatment controls (Figure 2-4B, lane 3). This result is consistent with G protein/receptor dissociation upon ligand activation, and is further evidence that the mPRα activates an inhibitory G protein. Pre-immune serum did not immunoprecipitate either mPRα (lane2) or G_i (lane 4), confirming the specificity of the antibody precipitation reactions.

cAMP response to 20β-S exposure

The demonstration that 20β-S treatment of G/T cells causes decreases in whole cell cAMP levels in a PTX-dependent manner would further support the proposed mechanism of progestin action through activation of an inhibitory G protein and the resulting decrease in adenylyl cyclase activity. The cAMP levels in the G/T cells were determined after treatment with 100 nM 20β-S for various time periods ranging from 1 to 30 minutes. Cells exposed to 20β-S showed a significant transient 5-fold decrease in cAMP levels at 10 minutes (one way ANOVA and Bonferroni's multiple comparison test $p < 0.01$, $n = 13$) which was observed in 5 separate experiments (Figure 2-5A). Pre-incubation with PTX, but not heat-inactivated PTX, blocked the inhibitory effect of 20β-S treatment on cAMP production by forskolin-treated G/T cells (one way ANOVA and Dunnett's multiple comparison test $*p < 0.05$, $**p < 0.001$, $n = 6$, Figure 2-5B). This PTX-dependent decrease in cAMP levels is consistent with progestin down-regulation of adenylyl cyclase activity through activation of an inhibitory G protein.

ERK activation by 20β-S exposure

Progestin activation of p42/44 MAP kinase (ERK) has been demonstrated in croaker oocytes, suggesting activation of a β/γ subunit G protein signaling pathway (Pace and Thomas, 2005b). To determine whether 20β-S causes activation of ERK in G/T cells, the cells were treated with 100 nM 20β-S and ERK activation was measured at various time points from 5 to 60 minutes (Figure 2-6). Western blotting showed that exposure of follicular cells to 20β-S resulted in an increase of ERK phosphorylation beginning at 10 minutes (Figure 2-6A). Mean ERK activation was increased 5 fold over initial (time 0) levels after 10 and 15 minutes treatment with 100 nM 20β-S (one way

ANOVA and Dunnett's multiple comparison on square root transformed data to remove variance inequality $p < 0.05$, $n = 3$) (Figure 2-6B). These results suggest that activation ERK is a potential signaling pathway through which progestins could inhibit apoptosis in G/T cells.

Modification of G/T cell death by progestins

In order to determine whether mPR activation by progestins mediates changes in serum starvation-induced death, cells were serum starved and treated with both natural and synthetic progestins. A decrease in the incidence of follicular cell death, assessed by trypan blue exclusion, was observed after 5-8 days of progestin treatment in G/T cultures obtained from ovaries containing a high percentage of fully grown oocytes (one way ANOVA and Dunnett's multiple comparison $p < 0.0001$, $n = 9$)(Figure 2-7A). In addition, treatment of the cells with R5020 at concentrations which should activate the nPR did not result in an alteration of cell death, indicating that this function of progestins in G/T cells is not mediated by the nPR. TUNEL staining of the G/T cells indicates that the number of cells which have undergone DNA cleavage is reduced by 20β-S but not by R5020 (one way ANOVA and Dunnett's multiple comparison $p < 0.001$, $n = 20$) (Figure 2-7B). These data suggest that progestin inhibition of cell death occurs via a progestin-induced decrease in the incidence of DNA fragmentation likely mediated by mPR activation.

Discussion

This study is the first to demonstrate mPRα localization in granulosa and theca cells of any vertebrate species suggesting that mPRα may mediate physiological functions of progestins in the ovarian follicle. The mPRα protein is expressed in the plasma membranes of cultured Atlantic croaker G/T cells and is associated with specific progestin binding characteristic of mPRα. Experiments also demonstrate that the mPR signals via activation of an inhibitory G protein to decrease intracellular cAMP in a pertussis toxin sensitive manner and rapidly increases ERK phosphorylation in croaker G/T cells which is consistent with progestin/mPR signaling in other systems. In addition, the current results show that progestins alter G/T apoptosis in fish ovarian follicle cells, suggesting this function of progestin is conserved in different vertebrate classes. The finding that specific mPRα agonists, but not nPR agonists, exert these effects provides initial evidence for a novel function of the mPR as the mediator of progestin inhibition of apoptosis in fish.

The results of the present study demonstrate the presence of a functional membrane progestin receptor on granulosa and theca cells in Atlantic croaker ovaries. The receptor on croaker G/T cells has a single high affinity binding site with a binding affinity for 20β-S (K_D 1.5 nM) almost the same as that for the seatrout ovarian receptor (K_D 1.7 nM) (Patiño and Thomas, 1990). Several lines of evidence are consistent with the identity of this receptor being mPR. The recombinant seatrout mPRα produced in a human breast cancer over expression system has a similar binding affinity for 20β-S with a K_D of 7.58 nM (Thomas et al., 2007). The saturation analysis shows that [^{3}H]20β-S rapidly associates with the G/T cell membrane progestin receptor and binding reaches equilibrium within 30 minutes, in agreement with the rapid binding kinetics observed

with recombinant seatrout mPRα (Thomas et al., 2007). Moreover, the specificity of binding to G/T cell membranes is specific for progestins and is the same as the steroid specificity of seatrout mPRα expressed in *E. coli* and mammalian cells (Thomas et al., 2007; Zhu et al., 2003b). The binding of [^{3} H]20β-S to croaker G/T cell membranes was significantly displaced by 20β-S and progesterone, and weakly displaced by testosterone whereas, R5020, estradiol-17β and cortisol were ineffective competitors as has been observed previously for recombinant seatrout mPRα (Thomas et al., 2007). In addition, two synthetic progestins, Organon 02-0 and Organon 0-13, which have relatively high binding affinities for recombinant human mPRα (unpublished observation), and are agonists on mPRα-mediated induction of oocyte maturation in zebrafish (Harris, 2007), were also effective competitors for 20β-S binding to G/T cells. In contrast, the steroid binding characteristics of the G/T cell membranes differ from those of nPR and PGMRC1, which were also identified by RT-PCR in croaker ovarian follicle cells. The nuclear receptor agonist, R5020, displays a much higher affinity for the cytosolic progestin receptor in G/T cells than it does for the progestin receptor on the cell membranes of these cells. Recombinant mammalian PGMRC1 and the partially purified protein display relatively low affinity binding to progestins, with K_Ds in the 200 to 300 nM range (Meyer et al., 1996; Peluso et al., 2001; Peluso et al., 2006), 30-40 times lower than the progestin binding affinity of the G/T membranes, and also bind cortisol with relatively high affinity (Cahill, 2007). Taken together, the current data strongly suggest that the progestin binding protein expressed on G/T cell membranes is mPRα and further suggests that any progestin activity initiated at the membrane surface is acting via the mPR.

In addition to G/T cell membranes displaying progestin binding characteristic of mPRα, rapid progestin-initiated G protein signaling in these cells is consistent with previously observed mPRα signaling. Exposure of cell membranes to 20β-S and the selective membrane receptor agonist Organon 02-0 but not the selective nuclear receptor agonist R5020 results in a significant increase in G protein activation, measured by the incorporation of a radiolabeled, non-hydrolysable form of GTP ([^{35}S] GTPγS) into the cell membrane. This suggests first, that 20β-S and Organon 02-0 bind a receptor on the cell membrane which can activate G proteins and second, that the receptor is not the nuclear receptor. mPR activation of G proteins has also been demonstrated by recombinant seatrout mPRα expressed in mammalian cells (Thomas et al., 2007), human mPR in myometrial cells (Karteris et al., 2006) and endogenous mPR in croaker sperm (Tubbs, 2007).

Progestin/mPRα activation of a pertussis toxin-sensitive inhibitory G protein in croaker G/T cells is consistent with previous studies examining the intracellular signaling pathways activated by mPRs. Antibodies directed against a suite of inhibitory alpha subunits ($G\alpha_{i,o,t,z}$) of heterotrimeric G protein complexes co-immunoprecipitated mPRα in croaker G/T cells indicating that mPRα is physically associated with an inhibitory G protein in these cells though it does not establish direct coupling between the two. These data agree with previous findings that recombinant seatrout mPRα expressed in mammalian cells and human mPRα expressed in immortalized cancer and myometrial cells are co-immunoprecipitated with inhibitory G proteins (Karteris et al., 2006; Thomas et al., 2007). Pertussis toxin inhibition of progestin membrane binding in G/T cells is additional evidence for a direct coupling between mPR and G proteins as PTX acts to

uncouple inhibitory alpha G protein subunits from their receptors. A decrease in progestin binding upon pertussis toxin exposure indicates that the association between the mPR and the G protein is necessary for maximal progestin binding and suggests a conformational change in the mPR structure in the absence of a G protein. PTX inhibition of progestin binding has also been demonstrated in the seatrout ovary (Pace and Thomas, 2005a), by recombinant seatrout mPRα and human mPR (Thomas et al., 2007).

The characteristics of progestin activation of second messenger signaling in croaker G/T cells are also consistent with the identity of the receptor mediating progestin actions being mPRα. Decreases in the intracellular levels of cAMP upon progestin exposure, presumably via a down regulation of adenylyl cyclase, by the inhibitory alpha G protein have been observed in multiple studies including those in seatrout oocytes (Pace and Thomas, 2005a), recombinant seatrout mPR, and mPR in immortalized cancer and myometrial cells (Karteris et al., 2006; Thomas et al., 2007). The sensitivity of progestin induced cAMP decreases to activated pertussis toxin in all of these studies more specifically indicates that the mPR is coupled to a $G\alpha_i$ subunit. Exposure of croaker G/T cells to 20β-S also increases p42/44 ERK activation presumably via the actions of the β/γ G protein subunits and is also in agreement with previous studies. Progestin induced activation of ERK via mPR activation has been demonstrated in studies on other teleost mPRs (Hanna et al., 2006; Zhu et al., 2003b). Taken together, these data demonstrating mPR association with a PTX- sensitive G protein, PTX sensitive decreases in intracellular cAMP and the activation of ERK indicate conservation of mPR signaling in a variety of female reproductive tissues.

mPRα expression on the plasma membranes of both granulosa and theca cells in a puncatate pattern consistent with its identity as a membrane localized steroid receptor is final evidence that the functional membrane localized progestin receptor in croaker G/T cells is the mPR. Immunocytochemical staining of the mPRα in co-cultures of granulosa and theca cells clearly reveals the presence of mPRα on the plasma membranes. mPRα appears to be expressed in both the elongated theca cells and the rounded granulosa cells indicating that the mPRα is present in both follicular cell types. Expression of mPRα in both follicular cell subtypes agrees with the description of biochemically isolated progesterone binding sites on plasma membranes from independently isolated granulosa and theca cells (Rae et al., 1998) and the expression of mPRα in both follicular cell types suggests a biological function of the mPR which involves both granulosa and theca cells as opposed to only one or the other. mPRα staining was seen on the plasma membranes of granulosa and theca cells and also intracellularly particularly around the nucleus. While there is not direct evidence, the intracellular staining of mPRα is likely mPR in the endoplasmic reticulum. Membrane proteins are trafficked to the plasma membrane via the endoplasmic reticulum and there are reports of G protein coupled receptors becoming "trapped" in the endoplasmic reticulum with only a small portion becoming fully active and trafficked to the plasma membrane (Petaja-Repo et al., 2000). Thus, it is not abnormal to see mPR staining on the endoplasmic reticulum. Immunocytochemistry also revealed punctate staining of the mPRα in follicular cells. Puncate staining of membrane receptors is often associated with localization of proteins to clathrin coated signaling rafts on the plasma membranes and is consistent with mPRα functioning as a G protein coupled receptor.

There are multiple potential functions of progestins in croaker G/T cells including regulation of follicular cell apoptosis and steroidogenesis. Progestin treatment has been shown to regulate steroidogenic enzyme expression and activity in several mammalian systems. Progestins increase P450SCC and 3β-HSD message in porcine granulosa cells (Rodway et al., 1999a; Rodway et al., 1999b; Swan et al., 2002) and may inhibit P450arom activity and estradiol production in rat granulosa cells (Fortune and Vincent, 1983; Schreiber et al., 1981). Treatment of granulosa and theca co-cultures from Atlantic croaker with 20β-S resulted in an increase in testosterone production over ethanol treated cells yet further investigation into this action of progestins in croaker G/T cells suggested that this was not due to a progestin receptor mediated action but rather due to conversion of progestin to testosterone by these cells (data not shown). Thus steroidogenic responses of croaker G/T cells to progestin were not explored further.

Exposure of croaker G/T cells to progestin was shown to inhibit follicular cell apoptosis. This data suggests that the anti-apoptotic action of progestin on follicle cells is a conserved action of progestins across multiple vertebrate orders although the mechanism of this progestin action may differ across vertebrates. The protective effects of progestin seen in other species around the periovulatory period occur in the teleost follicle when a high percentage of the follicles in the ovary are fully grown and ready to undergo final oocyte maturation and release. Croaker are batch spawners and fully grown follicles may be protected from apoptosis in the presence of 20β-S just prior to spawning allowing for the synchronization of oocyte maturation and enabling the fish to spawn a large number of fertilizable eggs. This phase of croaker ovarian development is similar to the periovulatory period in mammals when the majority of progesterone's

protective effects on granulosa cells have been seen (Chaffin and Stouffer, 2000; Engmann et al., 2006; Quirk et al., 2004; Rueda et al., 2000; Svensson et al., 2000; Telleria et al., 1999). The introduction of progestin to these cells *in vivo* would signal the proximity of spawning as 20β-S levels remain extremely low, if not completely absent, until just prior to spawning. Interestingly, apoptosis of the ovarian follicle has been shown to be critical in the re-absorption of the post-ovulatory follicle (Drummond et al., 2000) as well as in the atresia of excess vertebrate oocytes (Johnson, 2003) and thus the absence or decline in progestins post spawning would result in apoptosis of the follicle and re-absorption of the oocytes.

While croaker G/T cells likely express three potential progestin receptors only the mPR exhibits the binding and signaling characteristics necessary for mediation of progestin's anti-apoptotic actions. The presence of mRNAs encoding mPRα, PGRMC1 and nPR suggest the expression of three potential progestin receptors in the teleost follicle: two novel membrane localized receptors and one nuclear receptor. Competition studies on cytosolic fractions confirm the presence of a functional nuclear receptor in the ovary of the croaker as has been previously described in the ovaries of closely related spotted seatrout (Pinter and Thomas, 1995). nPR expression in the follicular cells of the ovary was expected as nuclear receptor appears to be responsible for ovulation of mature oocytes from the teleost ovarian follicle (Pinter and Thomas, 1999). The nPR is expressed by both granulosa and theca cells in the human (Maybin and Duncan, 2004), and the dog (Vermeirsch et al., 2001) and bovine granulosa/luteal cells (Van den Broeck et al., 2002) as well as those of rats (Natraj and Richards, 1993), primates (Duffy et al., 1997) and humans (Misao et al., 1998a; Misao et al., 1998b). RT-PCR using primers

designed against zebrafish PGRMC1 identified a product of predicted size (~400bp) in G/T cell mRNA suggesting the presence of PGRMC1 in these cells. PGRMC1, a 28kD single transmembrane protein was first characterized in sperm (Falkenstein et al., 1999) and subsequently characterized in rat spontaneously immortalized granulosa cell (SIGC) membranes (Peluso et al., 2001; Peluso et al., 2006). PGRMC1 has also been found in cultured human granulosa/luteal cells (Engmann et al., 2006) and rainbow trout (Mourot et al., 2006), where it appears to be expressed in the follicle cells as well as in zebrafish oocytes (Harris, 2007). While the current study does not examine interactions between the three potential progestin receptors the co-expression of mPRα, PGRMC1 and nPR in croaker G/T cells during the period of gonadal recrudescence suggests the possibility of cross-talk between these three receptors while progestin levels are high. While there is evidence of mPR crosstalk with the nPR there is no published evidence that mPRs interact directly with PGRMC1. Evidence of mPR crosstalk with the nPR is seen in human myometrial cells where mPR mediates functional progesterone withdrawal just prior to birth (Karteris et al., 2006). In myometrial cells mPR activation decreases the expression of nPR coactivators necessary for nPR transcriptional activity.

The protective function of progestin in croaker granulosa and theca cells is initiated by the endogenous progestin at low nanomolar concentrations of 20β-S and a selective human mPR agonist Organon 02-0 (which binds to mPR in Atlantic croaker) but not by R5020 or 17,20β-P at concentrations which should activate the nuclear receptor but not the membrane receptor indicating that this action is not initiated by the nPR. Additionally, the presence of a single progestin binding site on follicular cell membranes with the characteristics of the mPR and the identification of mPRα on the

plasma membranes of the follicle cells indicates that mPR is the only functional progestin receptor in these cell membranes and suggests that PGRMC1, while possibly involved, is not the primary progestin receptor initiating 20β-S's protective effects against serum starvation-induced cell death. The manner of cell death inhibited by progestins is controlled and not necrotic in nature as indicated by TUNEL staining. The pattern of cell death determined by TUNEL staining is the same as the pattern determined by trypan blue exclusion confirming that the decrease in cell death is due to a decrease in the incidence of DNA cleavage, a controlled cell death process, and not an uncontrolled necrotic process. A potential mechanism for mPR inhibition of apoptosis is mPR activation of ERK and Akt. The current study shows progestin activation of ERK in G/T cells and progestin has been shown to activate ERK in the oocyte (Pace and Thomas, 2005b) and mPR has been shown to activate ERK in human cell lines stabling expressing spotted seatrout mPRα (Zhu et al., 2003b) and zebrafish mPRα and mPRβ (Hanna et al., 2006). Progestin has also been shown to activate Akt in croaker oocytes (Pace and Thomas, 2005b). Both ERK and Akt activation have been shown to both directly and indirectly inhibit apoptosis in mammals (Song et al., 2005). Activated ERK up-regulates the expression of anti-apoptotic members (Lin et al., 2002) and inactivates the pro-apoptotic members (She et al., 2002) of the Bcl-2 family of proteins. Akt directly inhibits apoptosis through inhibition of the pro-apoptotic Bcl-2 family member BAD (Datta et al., 1997) and caspase 9 (Cardone et al., 1998), an upstream mediator of apoptosis. While mPR activation of ERK and Akt suggest a possible mechanism for mPR inhibition of apoptosis further studies are needed to gain a fuller understanding of the link between mPR activation and inhibition of cell death.

Conclusions

The data presented in this study are the first to demonstrate mPR expression, membrane localization and signaling in the ovarian follicle of any vertebrate species. In addition, mPR activation of an inhibitory G protein, decreases in cAMP and activation of ERK appear to be conserved in female vertebrate reproductive tissues. This study also demonstrates that inhibition of apoptosis in the ovarian follicle is also a conserved function of progestins in teleost fish. This work is also the first to suggest a novel anti-apoptotic role for the mPR in the ovarian follicle and to link the mPR to apoptotic processes. Additional studies are needed to elucidate the signaling mechanism leading from progestin activation of mPR to inhibition of DNA cleavage and apoptosis and to examine the potential interaction of the three progestin receptors in ovarian follicular apoptosis.

Literature Cited

Ashley, R. L., Clay, C. M., Farmerie, T. A., Niswender, G. D., and Nett, T. M. (2006). Cloning and characterization of an ovine intracellular seven transmembrane receptor for progesterone that mediates calcium mobilization. Endo *147*, 4151-4159.

Benninghoff, A. D., and Thomas, P. (2006). Gonadotropin regulation of testosterone production by primary cultured theca and granulosa cells of Atlantic croaker: I. Novel role of CaMKs and interactions between calcium- and adenylyl cyclase-dependent pathways. Gen Comp Endocrinol *147*, 276-287.

Cahill, M. (2007). Progesterone receptor membrane component 1: An integrative review. Steroid Biochem Mol Bio *105*, 16-36.

Cai, Z., and Stocco, C. O. (2005). Expression and regulation of progestin membrane receptors in the rat corpus luteum. Endocrinology *146*, 5522-5532.

Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998). Regulation of cell death protease caspase-9 by phosphorylation. Science *282*, 1318-1321.

Chaffin, C. L., and Stouffer, R. L. (2000). Role of gonadotrophins and progesterone in the regulation of morphological remodelling and atresia in the monkey peri-ovulatory follicle. Hum Reprod *15*, 2489-2495.

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*, 231-241.

Drummond, C. D., Bazzoli, N., Rizzo, E., and Sato, Y. (2000). Postovulatory follicle: a model for experimental studies of programmed cell death or apoptosis in teleosts. J Exp Zool *287*, 176-182.

Duffy, D. M., Wells, T. R., Haluska, G. J., and Stouffer, R. L. (1997). The ratio of progesterone receptor isoforms changes in the monkey corpus luteum during the luteal phase of the menstrual cycle. Biol Reprod *57*, 693-699.

Engmann, L., Losel, R., Wehling, M., and Peluso, J. (2006). Progesterone regulation of human granulosa/luteal cell viability by an RU468-independent mechanism. J Clin Endo Meta *91*, 4962-4968.

Falkenstein, E., Heck, M., Gerdes, D., Grube, D., Christ, M., Weigel, M., Buddhikot, M., Meizel, S., and Wehling, M. (1999). Specific progesterone binding to a membrane protein and related nongenomic effects on Ca2+-fluxes in sperm. Endocrinology *140*, 5999-6002.

Fortune, J. E., and Vincent, S. E. (1983). Progesterone inhibits the induction of aromatase activity in rat granulosa cells in vitro. Biol Reprod *28*, 1078-1089.

Freshney, R. I. (1994). Culture of Animal Cells: A manual of basic technique. In (New York, Wiley-Liss).

Hanna, R., Pang, Y., Thomas, P., and Zhu, Y. (2006). Cell-surface expression, progestin binding, and rapid nongenomic signaling of zebrafish membrane progestin receptors α and β in transfected cells. J Endo *190*, 247-260.

Harris, C. (2007) Examination of the role of different classes of progesterone receptors in oocyte maturation in zebrafish, Danio rerio, Masters of Science, University of Texas, Port Aransas.

Johnson, A. L. (2003). Intracellular mechanisms regulating cell survival in ovarian follicles. Animal Repro Sci *78*, 185-201.

Josefsberg Ben-Yehoshua, L., Lewellyn, A., Thomas, P., and Maller, J. (2007). The role of *Xenopus* membrane progesterone receptor β in mediating the effect of progesterone on oocyte maturation. Mol Endocrinol *21*, 664-673.

Karteris, E., Zervou, S., Pang, Y., Dong, J., Hillhouse, E. W., Randeva, H. S., and Thomas, P. (2006). Progesterone signaling in human myometrium through two novel membrane G protein coupled receptors: potential role in functional progesterone withdrawal at term. Mol Endocrinol.

Kaslow, H. R., Lim, L. K., Moss, J., and Lesikar, D. D. (1987). Structure-activity analysis of the activation of pertussis toxin. Biochemistry *26*, 123-127.

Kazeto, Y., Goto-Kazeto, R., Thomas, P., and Trant, J. M. (2005). Molecular characterization of three forms of putative membrane-bound progestin receptors and their tissue-distribution in channel catfish, *Ictalurus punctatus*. J Mol Endocrinol *34*, 781-791.

Lin, H., Chen, C., Li, X., and Chen, B. D. (2002). Activation of the MEK/MAPK pathway is involved in bryostatin1-induced monocytic differenciation and up-regulation of X-linked inhibitor of apoptosis protein. Exp Cell Res *272*, 192-198.

Lyons, T. J., Villa, N. Y., Regalla, L. M., Kupchak, B. R., Vagstad, A., and Eide, D. J. (2004). Metalloregulation of yeast membrane steroid receptor homologs. Proc Natl Acad Sci U S A *101*, 5506-5511.

Maybin, J. A., and Duncan, W. C. (2004). The human corpus luteum: which cells have progesterone receptors? Reproduction *128*, 423-431.

Meyer, C., Schmid, R., Scriba, P. C., and Wehling, M. (1996). Purification and partial sequencing of high-affinity progesterone-binding site(s) from porcine liver membranes. Eur J Biochem *239*, 726-731.

Misao, R., Nakanishi, Y., Fujimoto, J., and Tamaya, T. (1998a). Steroid receptor mRNA levels in human corpus luteum. Horm Res *50*, 155-159.

Misao, R., Nakanishi, Y., Iwagaki, S., Fujimoto, J., and Tamaya, T. (1998b). Expression of progesterone receptor isoforms in corpora lutea of human subjects: correlation with serum oestrogen and progesterone concentrations. Mol Hum Reprod *4*, 1045-1052.

Moore, M. R., Spence, J. B., Kiningham, K. K., and Dillon, J. L. (2006). Progestin inhibition of cell death in human breast cancer cell lines. J Steroid Biochem Mol Biol *98*, 218-227.

Mourot, B., Nguyen, T., Fostier, A., and Bobe, J. (2006). Two unrelated putative membrane-bound progestin receptors, progesterone membrane receptor component 1 (PGMRC1) and membrane progestin receptor (mPR) beta, are expressed in the rainbow trout oocyte and exhibit similar ovarian expression patterns. Repro Bio Endo *4*.

Mussche, S., and D'Herde, K. (2001). Contribution of progesterone, follicle stimulating hormone and glucocorticoids in survival of serum-free cultured granulosa cell explants. J Endo *169*, 321-331.

Natraj, U., and Richards, J. (1993). Hormonal regulation, localization, and functional activity of the progesterone receptor in granulosa cells of rat preovulatory follicles. Endocrinology *133*, 761-769.

Pace, M. C., and Thomas, P. (2005a). Activation of a pertussis toxin-sensitive, inhibitory G-protein is necessary for steroid-mediated oocyte maturation in spotted seatrout. Dev Biol *285*, 70-79.

Pace, M. C., and Thomas, P. (2005b). Steroid-induced oocyte maturation in Atlantic croaker (Micropogonias undulatus) is dependent on activation of the phosphatidylinositol 3-kinase/Akt signal transduction pathway. Biol Reprod *73*, 988-996.

Patiño, R., and Thomas, P. (1990). Characterization of membrane receptor activity for 17α,20β,21-tryhydroxy-4-pregnen-3-one in ovaries of spotted seatrout (*Cynoscion nebulosus*). Gen Comp Endocrinol *78*, 204-217.

Peluso, J., Fernandez, G., Pappalardo, A., and White, B. (2001). Characterization of a putative membrane receptor for progesterone in rat granulosa cells. Bio Rep *65*, 94-101.

Peluso, J., Pappalardo, A., Losel, R., and Wehling, M. (2006). Progesterone membrane receptor component 1 expression in the immature rat ovary and its role in mediating progesterone's antiapoptotic action. Endocrinology *147*, 3133-3140.

Petaja-Repo, U. E., Hogue, M., Laperriere, A., Walker, P., and Bouvier, M. (2000). Export from the endoplasmic reticulum represents the limiting step in the maturation and cell surface expression of the human delta opioid receptor. J Biol Chem *275*, 13727-13736.

Pinter, J., and Thomas, P. (1995). Characterization of a progestogen receptor in the ovary of the spotted seatrout, Cynoscion nebulosus. Biol Reprod *52*, 667-675.

Pinter, J., and Thomas, P. (1997). The ovarian progestogen receptor in the spotted seatrout, *Cynoscion nebulosus*, demonstrates steroid specificity different from the progesterone receptors in other vertebrates. J Steroid Biochem Molec Biol *60*, 113-119.

Pinter, J., and Thomas, P. (1999). Induction of ovulation of mature oocytes by the maturation-inducing steroid 17,20β,21-trihydroxy-4-pregnen-3-one in the spotted seatrout. Gen Comp Endocrinol *115*, 200-209.

Quirk, S., Cowan, R., and Harman, R. (2004). Progesterone receptor and the cell cycle modulate apoptosis in granulosa cells. Endocrinology *145*, 5033-2043.

Rae, M. T., Menzies, G. S., and Bramley, T. A. (1998). Bovine ovarian non-genomic progesterone binding sites: presence in follicular and luteal cell membranes. J Endo *159*, 413-427.

Rodway, M. R., Swan, C. L., Crellin, N. K., Gillio-Meina, C., and Chedrese, P. J. (1999a). Steroid regulation of progesterone synthesis in a stable porcine granulosa cell line: a role for progestins. J Steroid Biochem Mol Biol *68*, 173-180.

Rodway, M. R., Swan, C. L., Gillio-Meina, C., Crellin, N. K., Flood, P. F., and Chedrese, P. J. (1999b). Regulation of steroidogenesis in jc-410, a stable cell line of porcine granulosa origin. Mol Cell Endocrinol *148*, 87-94.

Rueda, B., Hendry, I., Hendry, W., Stormshak, F., Slayden, O., and Davis, J. (2000). Decreased progesterone levels and progesterone receptor antagonists promote apoptotic cell death in bovine luteal cells. Bio Rep *62*, 269-276.

Schreiber, J. R., Nakamura, K., and Erickson, G. F. (1981). Progestins inhibit FSH-stimulated granulosa estrogen production at a post-cAMP site. Mol Cell Endocrinol *21*, 161-170.

Scott, A. P., Sheldrick, E. L., and Flint, A. P. F. (1982). Measurement of 17α,20β-dihydroxy-4-pregnen-3-one in plasma of trout (*Salmo gairdneri* Richardson): seasonal changes and response to salmon pituitary extract. Gen Comp Endocrinol *46*, 444-451.

She, Q. B., Ma, W. Y., Zhong, S., and Dong, Z. (2002). Activation of JNK1, RSK2, and MSK1 is involved in serine 112 phosphorylation of Bad by ultraviolet B radiation. J Biol Chem *277*, 24039-24048.

Song, G., Ouyang, G., and Bao, S. (2005). The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med *9*, 59-71.

Svensson, E. C., Markstrom, E., Andersson, M., and Billig, H. (2000). Progesterone receptor-mediated inhibition of apoptosis in granulosa cells isolated from rats treated with human chorionic gonadotropin. Biol Reprod *63*, 1457-1464.

Swan, C. L., Agostini, M. C., Bartlewski, P. M., Feyles, V., Urban, R. J., and Chedrese, P. J. (2002). Effects of progestins on progesterone synthesis in a stable porcine granulosa cell line: control of transcriptional activity of the cytochrome p450 side-chain cleavage gene. Biol Reprod *66*, 959-965.

Tang, Y. T., Hu, T., Arterburn, M., Boyle, B., Bright, J. M., Emtage, P. C., and Funk, W. D. (2005). PAQR proteins: a novel membrane receptor family defined by an ancient 7-transmembrane pass motif. J Mol Evol *61*, 372-380.

Telleria, C. M., Stocco, C. O., Stati, A. O., and Deis, R. P. (1999). Progesterone receptor is not required for progesterone action in the rat corpus luteum of pregnancy. Steroids *64*, 760-766.

Thomas, P., and Das, S. (1997). Correlation between binding affinities of C21 steroids for the maturation-inducing steroid membrane receptor in spotted seatrout ovaries and their agonist and antagonist activities in an oocyte maturation bioassay. Biol Reprod *57*, 999-1007.

Thomas, P., Pang, Y., Dong, J., Groenen, P., Kelder, J., de Vlieg, J., Zhu, Y., and Tubbs, C. (2007). Steroid and G protein binding characteristics of the seatrout and human progestin membrane receptor alpha subtypes and their evolutionary origins. Endocrinology *148*, 705-718.

Tokumoto, M., Nagahama, Y., Thomas, P., and Tokumoto, T. (2006). Cloning and identification of a membrane progestin receptor in goldfish ovaries and evidence it is an intermediary in oocyte meiotic maturation. Gen Comp Endocrinol *145*, 101-108.

Tubbs, C. (2007) Mechanisms of progestin–stimulated sperm hypermotility in two teleosts: the Atlantic croaker (*Micropogonias undulatus*) and the southern flounder (*Platylicthys lethigstomata*), Ph. D., The University of Texas, Port Aransas.

Van den Broeck, W., D'haeseleer, M., Coryn, M., and Simoens, P. (2002). Cell-specific distribution of progesterone receptors in the bovine ovary. Reprod Dom Anim *37*, 314-320.

Vermeirsch, H., Simoens, P., Coryn, M., and Van den Broeck, W. (2001). Immunolocalization of progesterone receptors in the canine ovary and their relation to sex steroid hormone concentrations. Reproduction *122*, 73-83.

Zhu, Y., Bond, J., and Thomas, P. (2003a). Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. Proceedings of the National Academy of Sciences of the United States of America *100*, 2237-2242.

Zhu, Y., Rice, C. D., Pang, Y. F., Pace, M., and Thomas, P. (2003b). Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proceedings of the National Academy of Sciences of the United States of America *100*, 2231-2236.

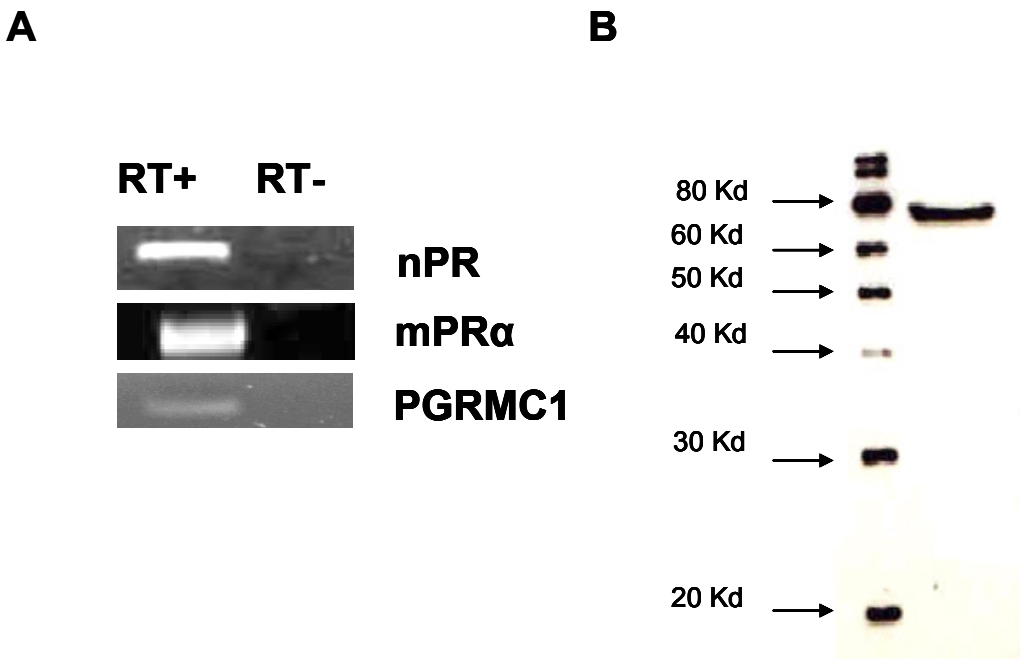

Figure 2-1: Progestin receptor expression in granulosa and theca co-cultures. A. nPR, mPRα and PGRMC1 mRNA expression using reverse transcription polymerase chain reaction on isolated follicular cells. RT plus and RT minus shown to confirm lack of DNA contamination. B. Western blotting in granulosa and theca cell plasma membranes using an antibody directed against an N terminal seatrout mPRα peptide showed a single immunoreactive band around 80kDa likely representing an mPRα dimer.

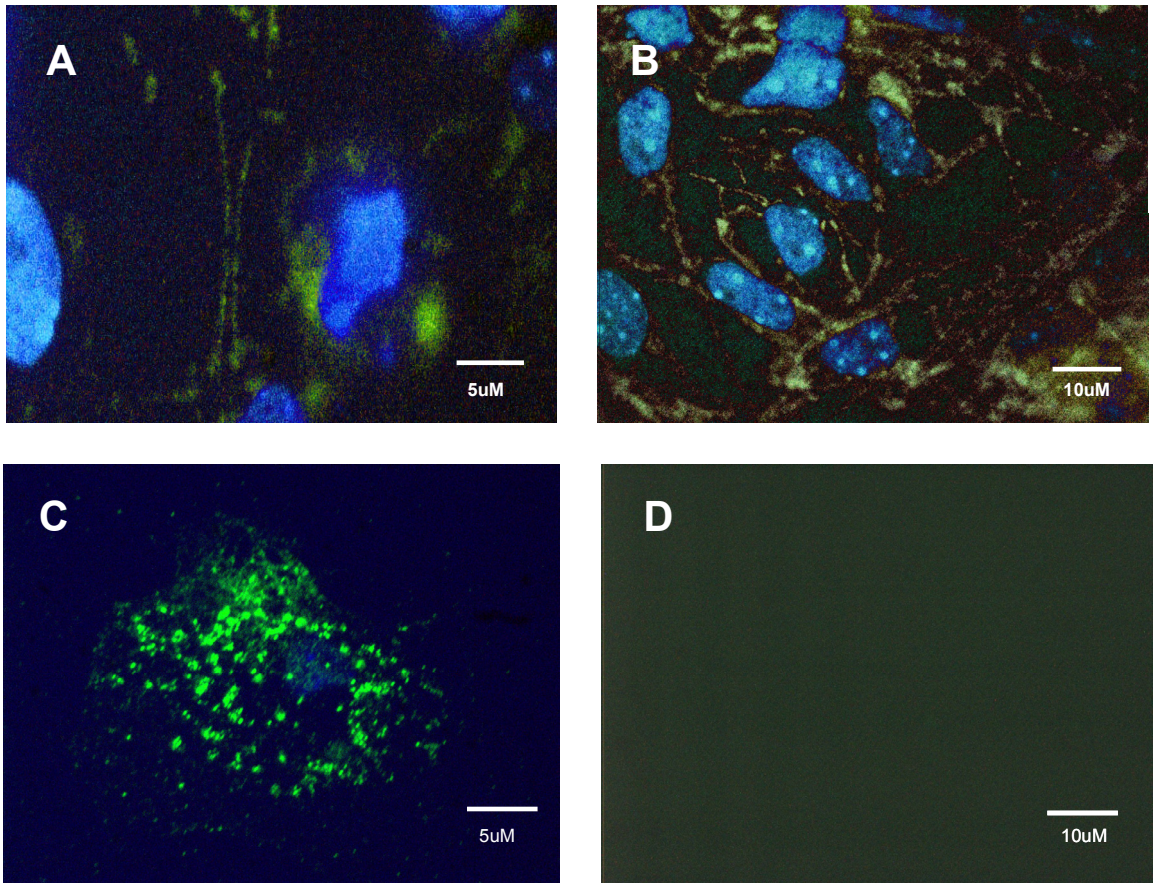

Figure 2-2: mPRα localization in granulosa and theca co-cultures. Immunocytochemistry of granulosa and theca cell cultures using an antibody directed against the N terminal region of the mPRα and DAPI nuclear staining showed mPRα expression on the plasma membrane (A) of both cell types (B) in a punctate pattern (C). Secondary antibody only (D).

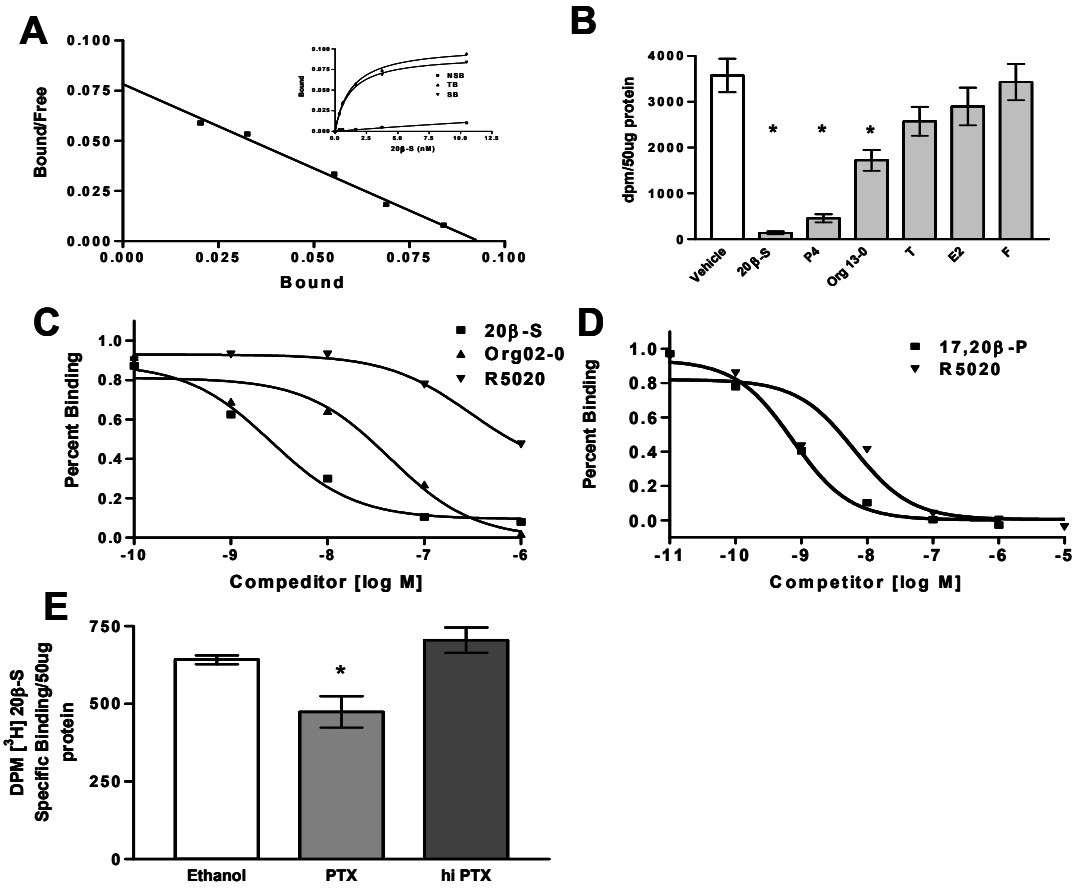

Figure 2-3: Characteristics of progestin binding in granulosa and theca cells. A. Representative saturation and Scatchard binding of radiolabled 20β-S to granulosa and theca co-culture plasma membranes. kD= 1.7nM 20β-S, Bmax= 0.0975nM or 0.39fmol/mg protein. B. Single point competition with 100nM competitor for 20β-S binding to plasma membranes of granulosa and theca cell cocultures. Data represents means ± SEM. Statistically significant differences from vehicle control were determined using one way ANOVA and Dunnett's multiple comparison test *p<0.001, n=3. Progesterone (P4), Testosterone (T), Estradiol (E2), cortisol (F). C. Competition with [^{3}H]20β-S for plasma membrane localized mPR binding by 20β-S (EC50=2.6nM), Organon 02-0 (EC50=44nM), and R5020 (EC50=297nM). Competition binding curves are generated from the average of three experiments. D. Competition with [3H]17,20β-P for cytosolic PR binding by 17,20β-P (EC50=0.73nM) and R5020 (EC50=6.3nM). Competition binding curves are generated from the average of three experiments. E. Effect of 1ug/mL pertussis toxin (PTX) or heat inactivated PTX (hiPTX) on [3H] 20β-S binding to membranes. Data represents means ± SEM from a representative replicate. Statistically significant differences from ethanol control were determined using one way ANOVA and Dunnett's multiple comparison test *p<0.05, n=3.

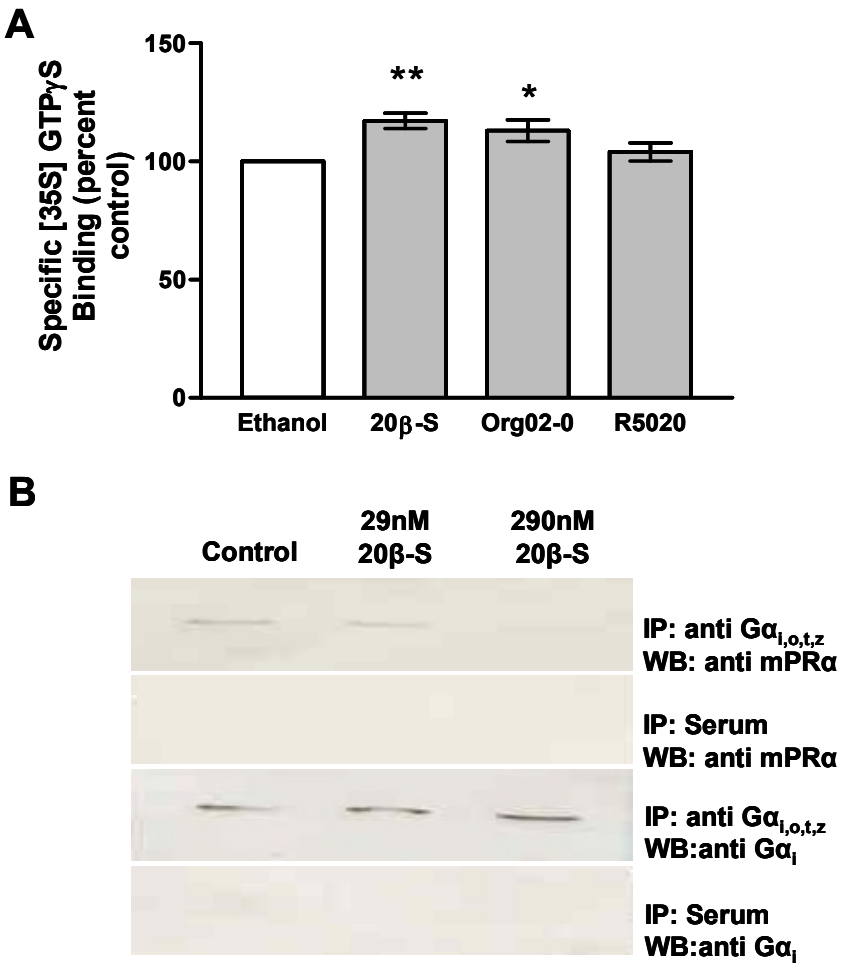

Figure 2-4: G protein activation in response to 20β-S exposure. A: Specific binding of [35S]GTPγS to plasma membranes of Atlantic croaker granulosa and theca cells in response to 20nM-100nM steroid. Data represents means ± SEM. Statistically significant differences from ethanol control were determined using one way ANOVA and Dunnett's multiple comparison test **$p<0.001$, $n=7$ *$p<0.05$, $n=3$ B: Semi-quantitative co-immunoprecipitation of mPR by anti $G\alpha_{i,o,t,z}$ in response to progestin. Representative blot.

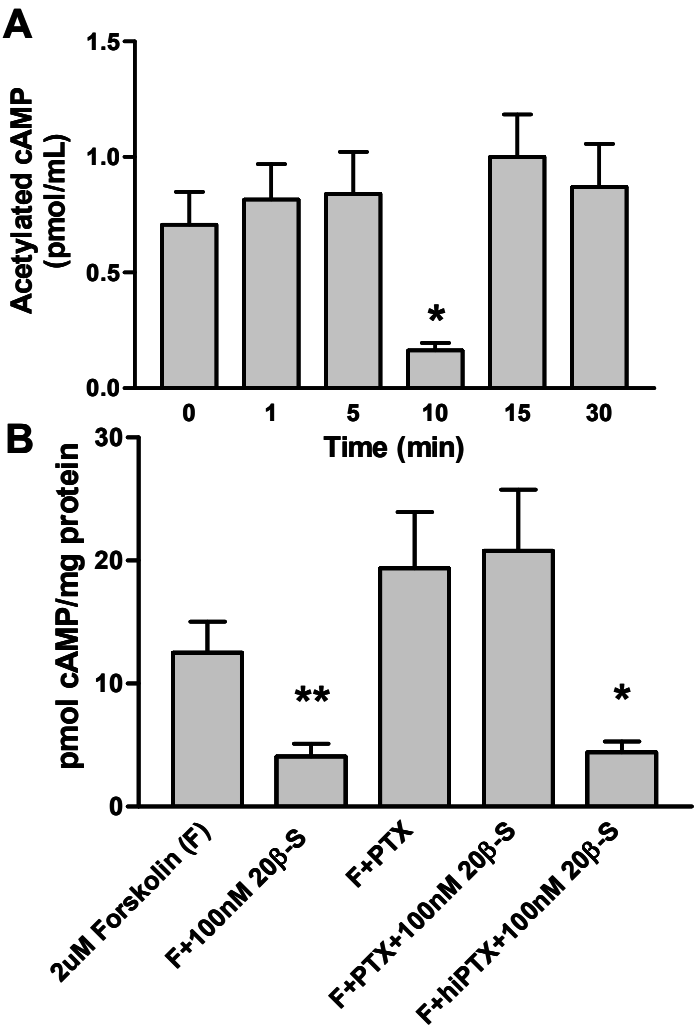

Figure 2-5: cAMP response to 20β-S exposure in cultured Atlantic croaker granulosa and theca cells. Cells were serum starved for 24 hours and pre-incubated with 100uM IBMX and 0.5ug/mL PTX or hiPTX (as indicated) for 15 minutes prior to progestin exposure. Cell extracts were collected at the indicated time points and assayed for cAMP by ELISA. (Cayman Chemical) A: Whole cell cAMP levels over 30 minutes following 100nM 20β-S exposure. Data represents means ± SEM. Statistically significant differences were determined using one way ANOVA and Bonferroni's multiple comparison test *p<0.01, n=13. B: Whole cell cAMP levels after 30 minutes exposure to 100nM 20-β-S with forskolin and PTX pretreatment. Data represents means ± SEM of log transformed data to remove variance inequality. Statistically significant differences from forskolin control were determined using one way ANOVA and Dunnett's multiple comparison test *p<0.05, **p<0.001, n=6.

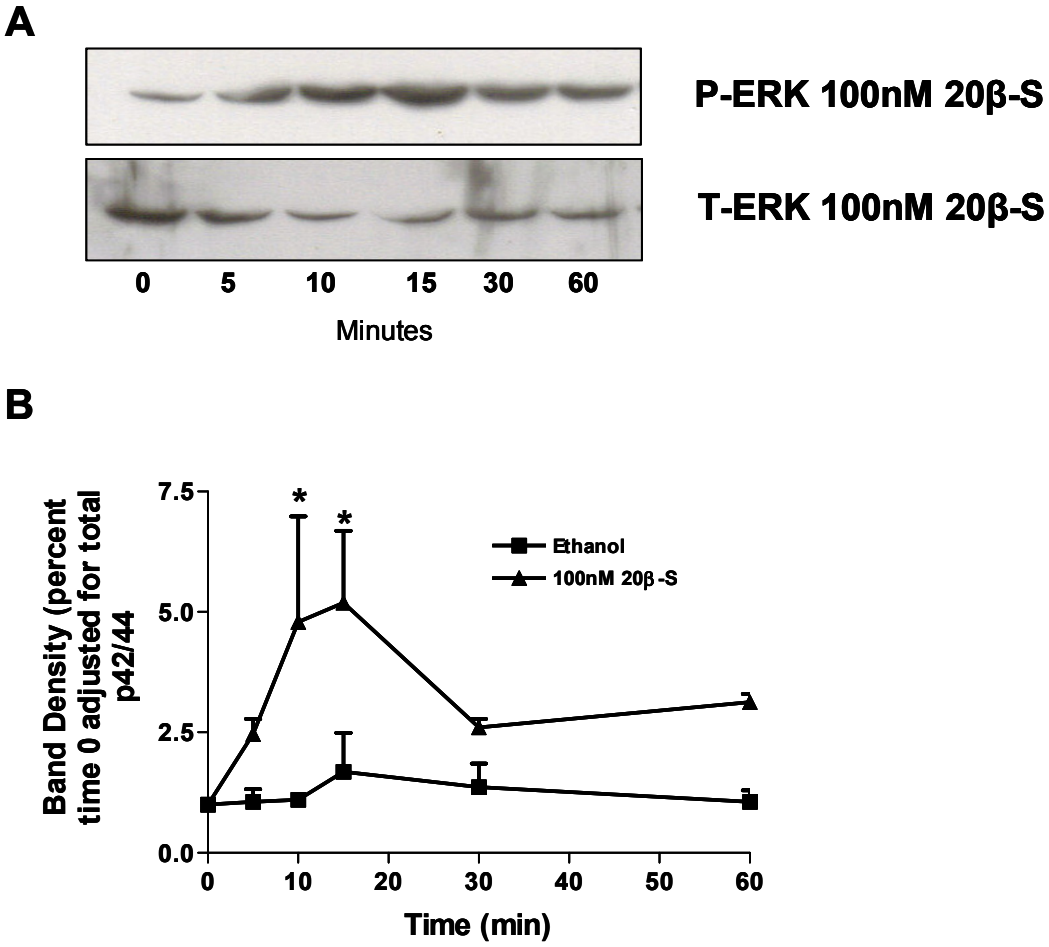

Figure 2-6: ERK activation in granulosa and theca co-cultures by 100nM 20β-S. A. Representative blot showing ERK phosphorylation upon 100nM 20β-S exposure. B. Band density shown as percent time 0 adjusted for total ERK protein of treated and control cell extracts. Data represents mean percent band density to time 0 adjusted to total p42/44 ± SEM. Statistically significant differences from time 0 were determined using one way ANOVA and Dunnett's multiple comparison test on square root transformed data *p< 0.05, n=3.

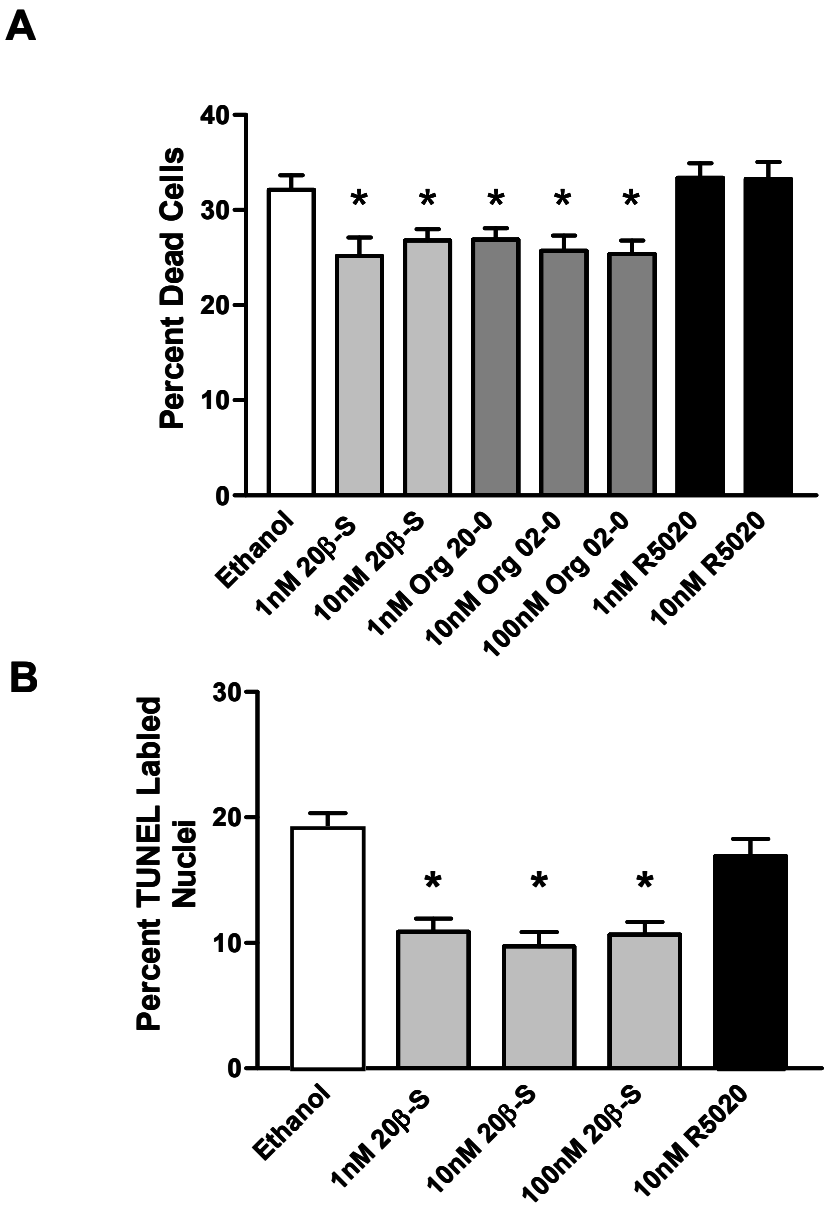

Figure 2-7: Modification of granulosa and theca cell death by progestins. A. Changes in the rate of granulosa and theca cell serum starvation induced death in response to progestins measured by trypan blue exclusion after 5-8 days exposure. Data represents means ± SEM. Statistically significant differences from ethanol control were determined using one way ANOVA and Dunnett's mulitple comparison test *p<0.0001, n=9. B. TUNEL stained nuclei in response to 6-7 days progestin exposure. Data represents means ± SEM. Statistically significant differences from ethanol control were determined using one way ANOVA and Dunnett's mulitple comparison test *p<0.001, n=20.

Chapter 3

Membrane progesterone receptor (mPR) expression and signaling in breast cancer cells and potential role in progestin inhibition of apoptosis

Summary

The expression, signaling and potential biological role of endogenous membrane progesterone receptors (mPRs) in SKBR3 and MDA-MB-468 breast cancer cell lines were evaluated. Quantitative PCR shows the presence of transcripts for mPRα, mPRβ and mPRγ in both cell lines with mPRα mRNA expression 10 times higher than that of mPRβ or mPRγ . Western blotting demonstrated the presence of mPRα, mPRβ and mPRγ proteins in plasma membrane fractions prepared from both cell lines. Western blotting also detected the presence of the nPR in SKBR3 cells but not in MDA-MB-468 cells. Progesterone bound to SKBR3 cell membranes with similar characteristics to those of mPRα shown in previous studies. The nuclear receptor agonist and antagonist, R5020 and RU486, did not compete for progesterone binding, indicating that the nPR is not the primary progesterone receptor expressed on SKBR3 cell membranes. Exposure of SKBR3 cells to progesterone resulted in rapid activation of an inhibitory G protein, a decrease in intracellular cAMP levels and p42/44 MAP kinase activation, consistent with progestin signaling through mPRs. Exposure of MDA-MB-468 cells to progesterone also resulted in rapid G protein activation. Treatment of serum starved SKBR3 cells and MDA-MB-468 cells with progesterone and the mPR agonist Organon 02-0 resulted in significant, dose-dependent decreases in cell death and DNA fragmentation, whereas testosterone, estradiol, dexamethasone, RU486 and R5020 treatments were ineffective.

Inhibition of cell death and DNA fragmentation was not accompanied by inhibition of caspase 3 activity, which suggests progesterone inhibits an alternative, caspase-independent death pathway. This is the first study to demonstrate expression and signaling of endogenously expressed mPRs in immortalized breast cancer cell lines. In addition, these results provide an initial indication that progesterone inhibition of apoptosis in these cells is mediated through mPRs. Involvement of mPRs in the development or progression of breast tumor growth through inhibition of cell death is an intriguing possibility and requires further investigation.

Introduction

Breast cancer is the second most common cancer in women and accounts for up to one third of cancer diagnoses in American women (American Cancer Society, ACS, 2003-2004 Breast Cancer Facts and Figures). Nearly 70% of breast cancers are steroid hormone-responsive and express either the nuclear estrogen or progesterone receptors (ACS Hormone Therapy). Because so many breast cancers proliferate in response to steroids, a wide variety of treatment options have been developed that block steroid action or synthesis in order to inhibit tumor growth.

Expression of the nuclear progesterone receptor (nPR) is often viewed as a marker of estrogen responsive malignancies, but a growing body of evidence indicates that progesterone receptor expression as merely an indication of estrogen-responsive growth may be an over simplification (Fuqua et al., 2005; Moore, 2004). Clinical evidence of progesterone's, in addition to estrogen's, effect on tumor development and growth was demonstrated in the Women's Health Initiative (WHI) Randomized Trial (Chlebowski et

al., 2003) which was terminated early due to an increased risk of breast cancer development exceeding the predefined stopping boundary. In addition, laboratory investigations have shown that progesterone causes proliferation of several immortalized breast cancer cell lines which express high levels of progesterone receptor and little or no estrogen receptor (Horwitz et al., 1978). Based on findings that progesterone has effects on breast cancer cell lines independent of estrogen, investigators have concluded that further studies are needed on the role of progesterone and progesterone receptors in breast cancer (Fuqua et al., 2005; Moore, 2004).

Nuclear steroid receptors are ligand-activated transcription factors that modulate the expression of a wide range of genes, including those regulating cell proliferation (Basu and Rowan, 2005; Ikeda and Inoue, 2004). Proliferation of cells mediated via nuclear steroid receptors through genomic mechanisms is a slow process requiring new mRNA and protein synthesis. However, rapid, non-nuclear signaling of progestins ultimately resulting in cell proliferation in immortalized breast cancer cell lines has also been reported. Studies examining progestin actions in breast cancer have shown that progestins are able to rapidly activate growth factor signaling pathways via direct, non-nuclear-mediated action through the nPR (Boonyaratanakornkit et al., 2001; Faivre et al., 2005; Lange et al., 1998; Lange et al., 1999; Skildum et al., 2005) and via cross- talk between the nPR and nuclear estrogen receptors (nER) (Ballare et al., 2003; Migliaccio et al., 1998; Vallejo et al., 2005) resulting in breast cancer cell growth and development of metastasis (Carnevale et al., 2007). Progestins also inhibit apoptosis of human breast cancer cells. While there is evidence that the nPR mediates progestin inhibition of apoptosis in several systems it is less clear in others, especially in breast cancer cell lines

which do not express the nPR. Medroxyprogesterone acetate, an nPR agonist, is able to inhibit serum starvation-induced apoptosis only in breast cancer cell lines which express the nPR (Ory et al., 2001) and the nPR antagonist RU486 is able to inhibit progestin protection from radiation induced apoptosis in T47D breast cancer cells (Vares et al., 2004). While these studies suggest nPR mediation of progestin's anti-apoptotic actions in these cells other studies examining progestin inhibition of apoptosis have found progestin response in an nPR negative cell line (Moore et al., 2006). In addition, the activation of Akt by progesterone appears to be necessary for progesterone's anti-apoptotic actions in MCF-7 cells but no direct link between Akt activation and the nPR was established (Alkhalaf et al., 2002). One possible mediator of progesterone actions, particularly in nPR negative cells, is the membrane progesterone receptor (mPR). The mPR was first identified, cloned and characterized in the ovaries of the *Cynoscion nebulosus* (Zhu et al., 2003b) and three isoforms (mPRα, mPRβ, and mPRγ) were subsequently cloned in humans (Zhu et al., 2003a). The human mPRs are seven-transmembrane proteins expressed on the plasma membrane of cells which bind progestins in a specific, displaceable, high affinity, limited capacity manner, characteristic of steroid membrane receptors (Thomas et al., 2007; Zhu et al., 2003a). In addition, mPRs activate G proteins in several cell types (Karteris et al., 2006; Thomas et al., 2007), although it is not a classical G protein coupled receptor (GPCR) but a member of the progestin and adipoQ receptor (PAQR) family (Lyons et al., 2004; Tang et al., 2005). The mPR expression has been shown in several breast cancer cell lines irrespective of nuclear steroid receptor expression. mPRs are expressed by MCF7 cells which express both the nPR and the nER as well as in SKBR3 cells which do not express

the nER but do express low levels of the nPR and in MDA-MB-231 cells which do not express either nER or nPR (Dressing and Thomas, 2007). Given that mPR is expressed in both nPR positive and negative cells, it is reasonable to suggest that mPR may mediate progestin's anti-apoptotic effects in the absence of the nPR and that mPR may be involved in progesterone's actions in the presence of nPR.

The objectives of this study were to examine the expression, signaling and biological function of mPRs in human breast cancer cell lines in order to asses their potential importance in breast cancer development and growth.

Methods

Chemicals

All chemicals were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted. Progesterone, estradiol, testosterone and cortisol were purchased from Steraloids (Newport, RI). RU486 was purchased from Sigma Aldrich and R5020 and [1,2,6,7,^{3}H (N)] progesterone were purchased from PerkinElmer (Waltham, MA). Antibodies for mPRα, mPRβ and mPRγ were generated by SigmaGenosis for Peter Thomas. mPRα polyclonal antibodies were generated against the peptide sequence TVDRAEVPPLFWKPC, mPRβ polyclonal antibodies were generated against the peptide sequence KILEDGLPKMPCTVC and mPRγ polyclonal antibodies were generated against the peptide sequence TDIKNDSYSWPMLC in rabbits. Antibodies directed against the nPR (Progesterone receptor Ab-8) were purchased from Lab Vision ThermoFisher (Fremont, CA). Integrin β-3 antibodies were purchased from Cell Signaling and β-actin (clone C-4) antibodies from MP Biomedical (Solon, OH). HRP-

linked secondary antibodies against rabbit and mouse were purchased from AbCam (Cambridge, MA) and Cell Signaling (Danvers, MA) respectively. p44/42 MAP kinase and Phospho-p44/42 MAP kinase antibodies were purchased from Cell Signaling (Danvers, MA).

Cell Culture

SKBR3 and MDA-MB-468 cells were obtained from American Type Culture Collection (Manassas, VA). SKBR3 cells were cultured in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 14 mM $NaCO_3$, penicillin/streptomycin/glutamine solution, gentamicin and 10% certified fetal bovine serum (FBS)(Gibco, Carlsbad, CA). MDA-MB-468 cells were cultured in Lebowitz-15 medium supplemented with 14 mM $NaCO_3$, penicillin/streptomycin/glutamine solution, gentamicin and 10% certified FBS. Media were changed every second day. Cultures were maintained in a humidified chamber at 37^{o}C with 5% CO_2 and sub-cultured using 0.05% trypsin with EDTA (Gibco, Carlsbad, CA) as necessary.

Real Time PCR

Cells were washed with PBS (136 mM NaCl, 2.7 mM KCl, 12.2 mM $NaPO_4$, 1.7 mM KH_2PO_4, pH 7.4) and harvested from 25 cm^2 flasks directly in TRI reagent (Sigma, St. Louis MO) and RNA was isolated as per manufacturer's instructions. Samples were DNase treated to remove any DNA using a DNA free RNA kit (Zymo Research, Orange CA). PCR was performed using Brilliant II SYBR Green QRT-PCR Mastermix 1-Step (Stratagene, Cedar Creek TX) for mPRα, mPRβ, mPRγ, (See Table 3-1 for primer sequences) on a Mastercycler ep realplex^2 (Eppendorf, Westbury, NY).

Preparation of Membranes

Cells were grown to 70-90% confluence in 150 cm^2 culture plates, washed with PBS and serum starved overnight. The cells were collected in 4 mL ice cold HAED (25 mM HEPES, 10 mM NaCl, 1 mM dithioerythritol, 1 mM EDTA, pH 7.6) with HALT protease inhibitor cocktail (Pierce, Rockford IL). Cells were sonicated for 10 seconds at a setting of 2.5 and centrifuged at 1000 X g for 7 minutes at 4^oC to remove unlysed cells and nuclear material. The supernatant was then centrifuged at 20,000 X g at 4^oC for 20 minutes to pellet the membrane fraction. The supernatant was carefully removed under suction and the pellet was resuspended with buffer for subsequent experimentation.

Western Blot Analysis for mPRs and nPR

Plasma membranes for mPRα, mPRβ, mPRγ and integrin western blotting were isolated as described above from SKBR3 and MDA-MB-468 cells, resuspended in PBS with HALT protease inhibitor cocktail and added in the ratio of 2:1 to 5X Lane Marker Reducing Sample Buffer (Pierce, Rockford IL) and loaded on a 10 % SDS-PAGE gel (15 μg protein/lane measured by the Bradford method). MagicMark Protein Ladder or BenchMark Pre-stained Protein Ladder (Invitrogen, Carlsbad CA) were run on each gel for protein size determination. SKBR3, MDA-MB-468 and MCF-7 cells were harvested for nPR and actin western blotting. Cells were harvested in PBS and centrifuged at 500 X g for 5 minutes to pellet the cells. Pellets were resuspended in 1 mL PBS containing protease inhibitor cocktail and lysed by sonication. Crude cytosolic extracts were obtained by centrifugation for 20 minutes at 20,000 X g to remove nuclear and membrane fractions. 50 μg of SKBR3 and MDA-MB-468 cell and 20 μg (to avoid nPR overloading) MCF-7 cell cytosolic extracts were loaded into sample wells. All samples were boiled for 10 minutes and cooled on ice prior to being run onto an 8% (for nPR) or

10% (for mPRs) SDS PAGE gel. The protein bands separated by electrophoresis were transferred onto nitrocellulose membranes. The membranes were blocked in PBS with 0.1% Tween-20 and 5% non-fat dry milk for 1 hour at room temperature. Membranes were washed in PBS three times for 5 minutes and incubated with primary antibody (dilution mPR antibodies-1:2500; dilution nPR antibody 1:10) in 5% non-fat dry milk overnight at 4^{o}C. After incubation with primary antibodies, membranes were washed three times for five minutes with TBS-T (20 mM Tris base, 150 mM NaCl, 0.1% Tween 20, pH 7.6) and incubated with an appropriate HRP-linked secondary antibody (1:2500) following the manufacturer's dilution recommendations at room temperature for one hour. Membranes were washed three times for five minutes with TBS-T. Supersignal WestPico was added (Pierce Rockford IL) to the membranes and membranes were exposed to Hyperfilm ECL (Amersham Piscataway NJ). Film was developed using Kodak developer and fixer (VWR, West Chester, PA).

MAP kinase activation assay

Cells were passed into 6-well cell culture plates (VWR, West Chester, PA) at $2x10^5$ cells per milliliter (three mL per well) and allowed to grow to 70% confluence. Cells were washed twice with PBS and fresh serum-free media were added. After 36 hours of serum starvation the cells were again washed twice with PBS and cultured with serum free media for 30 minutes prior to adding progesterone (final concentration: 100 nM in ethanol) or an equal volume (< 0.1% of total media volume) of ethanol carrier. The cells were incubated with progesterone for 5, 10, 15, 30 or 60 minutes and the incubation was terminated by rapidly removing the media by suction and followed by washing the cells with ice-cold PBS. Cells were scraped into 100 µL 1X Lane Marker

Reducing Sample Buffer (Pierce, Rockford IL). Samples were prepared for western blotting by sonication at a setting of 2.5 for 5 seconds and boiled for 10 minutes prior to loading 20 μL sample/well onto a 10% SDS PAGE gel. Following separation, proteins were transferred onto nitrocellulose membranes. The membranes were blocked in TBS-T and 5% non-fat dry milk for 1 hour at room temperature. Primary antibody was added (1:1000) and membranes were incubated overnight at 4^{o}C with gentle agitation. After the primary antibody incubation, membranes were washed, incubated with an HRP-linked goat anti-rabbit secondary antibody (1:2500), and visualized as described above.

Radiolabled GTPγS binding and immunoprecipatation

Cells were grown to confluence in 150 cm^2 culture dishes. Cells were washed twice with PBS and serum starved for 12 hours prior to membrane preparation. For the binding assay, the membranes were isolated following the protocol described previously and resuspended in binding buffer (100 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.6 mM EDTA, 0.1% BSA, 50 mM Tris-HCl, pH 7.6) with protease inhibitor cocktail (Sigma, St. Louis MO) to a concentration of 0.5 mg/mL. 250 μL membrane re-suspension was added to an equal volume of 1 nM [^{35}S] GTPγ-S and pre-incubated with 10 μM GDP in the presence or absence of 500 nM unlabeled GTPγ-S (BioMol, Plymouth Meeting, PA) at room temperature for 5 minutes. Steroids or ethanol of various concentrations were added at the end of the pre-incubation period and the mixture was incubated with gentle agitation for a further 15 minutes at room temperature. The binding experiment was terminated by rapid filtration of triplicate 100 μL reaction volumes onto Whatmann GF/B filters presoaked in BSA-free binding buffer and washed twice with 4 mL of the same buffer. For the immunoprecipitation experiment the membranes were resuspended in

binding buffer with protease inhibitor cocktail to a concentration of 2 mg/mL. 500 μL of the membrane suspension was added to an equal volume of 8 nM [^{35}S] GTPγ-S and pre-incubated with 10 μM GDP and in the presence or absence of 500 nM unlabeled GTPγ-S (BioMol, Plymouth Meeting, PA) at room temperature for 5 minutes. Steroids or ethanol of various concentrations were added to 200 μL aliquots of the reaction mixtures at the end of the pre-incubation period and the samples were incubated with gentle agitation for a further 30 minutes at 4^{o}C. Binding was terminated with the addition of 750 μL ice-cold 100 μM GDP/GTPγ-S in binding buffer to the 200 μL reaction volume. All the following procedures were completed at 4^{o}C. Membranes were pelleted using centrifugation at 20,000 X g for 30 minutes and resuspended in 200 μL solubilization buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 25 mM Tris-HCl, pH 7.4) with fresh protease inhibiting cocktail and incubated with gentle rotation for 2 hours. Antibodies directed against various G proteins were added at a concentration of 1:200 (Santa Cruz, Santa Cruz CA) and samples were incubated over-night with rotation. The following day samples were again centrifuged at 20,000 X g for 30 minutes and supernatants were added to 25 μL Protein A/G beads and incubated with gentle rotation for an additional 2 hours. Beads were washed three times with wash buffer (50 mM HEPES, 100 μM NaF, 50 mM sodium phosphate, 100 mM NaCl, 1% Triton X-100, 0.1% SDS, pH 7.4) and boiled in 100 μL PBS with 0.5% SDS for 10 minutes. Radioactivity was counted on a scintillation counter (Beckmann LS6000SC) for both the binding and the immunoprecipitation experiments.

cAMP measurement

Cells were grown to approximately 70% confluence in 6-well culture plates. Prior to experimentation the cells were washed twice with PBS and then serum starved for 36 hours. Cells were washed with PBS and pretreated with serum-free media containing 10 μM IBMX dissolved in DMSO for 20-30 minutes. Progesterone at a final concentration of 100 nM or ethanol vehicle (< 0.1% media volume) were added and incubated for 0, 1, 5, 10, 15 or 30 minutes. At the end of the incubation period, the media was quickly removed and 100 μL 0.1M HCl was added to each well. After 20 minutes treatment with HCl the cells were scraped off the plates, collected and stored at -20 °C until assayed by ELISA in duplicate according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

Cell Death Assay

Cell death assays protocols were adapted from previous studies (Moore et al., 2000; Moore et al., 2006). Briefly, cells were grown in 25 cm^2 flasks. Upon 100% confluence the cells were washed twice with PBS and fresh, serum free media with various steroid concentrations was added to duplicate or triplicate flasks. The cells were incubated from four to seven days with no medium changes. Steroid was supplemented every second day with the addition of 1μL ethanol containing steroid per 3.5 mL media. Upon conclusion of the experiment, the incubation media was removed and placed into a 15mL tube. The adherent cells were harvested by washing them twice with 2 ml Hank's saline solution, followed by incubating them for 5 minutes in 2 mL Hank's saline. Hank's saline solution from the washes and Hank's saline solution containing adherent cells was added to the media from the incubation and centrifuged at 1000 X g for 5 minutes to pellet all cells. Cells were resuspended in 250 μL Hank's saline. 50 μL 0.4%

filtered Trypan Blue Stain was added and the cells were incubated for 5 minutes. Cells were loaded onto a hemocytometer and viability was determined by Trypan Blue Stain exclusion. A total of 500 cells from each flask were scored for viability. All experiments were repeated at least three times. Some data was square root transformed to remove differences in variance as indicated.

TUNEL assay

Cells were grown in 25 cm^2 flasks. Upon reaching 100% confluence the cells were washed twice with PBS and fresh serum free media with various steroid concentrations were added as described above. The cells were incubated for 24-48 hours and harvested in Hank's saline solution as described above. TUNEL was performed according to the manufacturer's instructions (Clontech, Mountain View, CA) using the ApoAlert DNA Fragmentation Assay. Apoptotic nuclei were counted using a Nikon Eclipse E600 fluorescent microscope as a proportion of total cells in 5 random fields of view for each flask.

Caspase 3 Activity

Cells were treated as in the TUNEL assay. Cells were harvested and caspase 3 activity was determined using Caspase-3 ApoAlert Assay plate (Clontech, Mountain View CA) as per manufacturer's instructions.

Statistical Analyses

One way ANOVA with either Dunnett's multiple comparison or Bonferroni's multiple comparison was used to determine statistical differences between control and experimental treatments using GraphPad Prism (San Diego, CA). Square root

transformations of the data were used as indicated in order to remove significant differences in variance.

Results

Identification of mPR and progesterone binding

QPCR shows the presence of mRNAs of all three mPR isoforms in both SKBR3 and MDA-MB-468 cell lines (Figure 3-1A). mPRα mRNA levels were 10 times higher then mPRβ or mPRγ in both cell lines. SKBR3 cells appear to express higher levels of mPRα and mPRγ than MDA-MB-468 cells while the MDA-MB-468 cells contain slightly higher levels of mPRβ mRNA.

Western blotting for the mPRs shows the presence of 80 kD mPRα and mPRβ dimers and a 40 kD mPRγ monomer in both SKBR3 and MDA-MB-468 cell membranes (Figure 3-1B). SKBR3 cells appear to express slightly more mPRα and mPRγ proteins than MDA-MB-468 cells while MDA-MB-468 cells have higher mPRβ expression which is consistent with the QPCR results. The nPR is weakly expressed by SKBR3 cells while no nPR was found in MDA-MB-468 cells (Figure 3-1C).

Saturation and Scatchard analyses of [^{3}H]-progesterone binding demonstrate the presence of a high affinity (K_D = 10.59 nM progesterone), limited capacity (Bmax = 0.33 nmol/mg protein), single progesterone binding site on SKBR3 membranes (Figure 3-1D). A single point competitive binding assay showed that 50 nM progesterone was very effective in displacing [^{3} H]-progesterone binding to the receptor (Figure 3-1E). Two synthetic progestins, Organon 02-0 and Organon 13-0, which have high binding affinities for the progestin receptor on croaker G/T cell membranes, were also relatively

effective competitors at a concentration of 50 nM for [^{3} H]-progesterone binding to the receptor on SKBR3 cell membranes. Testosterone (50 nM) caused slight displacement of [^{3} H]-progesterone binding, whereas the nuclear progesterone receptor agonist and antagonist R5020 and RU486, and cortisol and estrogen were ineffective as competitors of [^{3}H] progesterone binding to SKBR3 membranes (Figure 3-1E).

mPR G protein signaling

Progesterone treatment (20 nM) caused significant increases in [^{35}S] GTPγS binding to both SKBR3 (Figure 3-2A) and MDA-MB-468 (Figure 3-2B) cell membranes. At a concentration of 100 nM, progesterone caused a 30% increase in [^{35}S] GTPγS binding to SKBR3 cell membranes over control values (one way ANOVA and Dunnett's multiple comparison *$p < 0.05$, **$p < 0.001$, $n = 4$ and Student's t-test *$p < 0.05$, $n = 3$ respectively). Immunoprecipitation experiments using solubolized SKBR3 membranes showed a significant (one way ANOVA Dunnett's $p < 0.05$, $n = 5$) 150% increase in the amount of [^{35}S] GTPγ-S immunoprecipitated by antibodies directed against inhibitory G proteins upon progesterone exposure and no significant increase in of [^{35}S] GTPγ-S immunoprecipitated by antibodies directed against stimulatory G proteins (Figure 3-2C) indicating selective progesterone activation of an inhibitory G protein.

mPR intracellular signaling

Exposure of SKBR3 cells to 100nM progesterone resulted in a significant (one way ANOVA Dunnett's multiple comparison $p < 0.05$, $n = 5$) reduction in intracellular cAMP levels compared to the control no treatment values at 5, 10 and 30 minutes (Figure 3-3A). Progesterone also caused a transient, significant (one way ANOVA and Dunnett's multiple comparison $p < 0.05$, $n = 4$) increase in p42/44 MAPK activation in SKBR3

cells between 5 and 30 minutes exposure which had disappeared by 60 minutes exposure (Figure 3-3B,C).

Progestin inhibition of serum starvation-induced death

Exposure of SKBR3 cells to 10nM progesterone resulted in a significant (one way ANOVA and Dunnett's multiple comparison on square root transformed data $p < 0.05$, $n = 6$) ~15% decrease in serum starvation-induced death assessed by trypan blue exclusion (Figure 3-4A) which was not replicated by the nPR agonist R5020, the nPR antagonist RU486, dexamethasone or estradiol-17β. Progesterone also significantly (one way ANOVA and Dunnett's multiple comparison $p < 0.01$, $n = 6$) inhibited serum starvation-induced cell death ~14% in MDA-MB-468 cells, whereas R5020, RU486, dexamethasone and estradiol-17β were ineffective (Figure 3-4B). Moreover, the progesterone-induced decrease in cell death in SKBR3 cells appeared to be concentration-dependent (Figure 3-5A) with a significant decrease (~15%) in cell death occurring at 10 nM (one way ANOVA and Dunnett's multiple comparison $p < 0.05$, $n = 9$), increasing to ~35% at 1000 nM progesterone ($p < 0.001$). In contrast, no concentration –dependent effects of progesterone on cell death in MDA-MB-468 cells were observed over the concentration range tested in this study (Figure 3-5B). Cell death was inhibited ~ 20% at a concentration of 1nM, the lowest progesterone concentration investigated (one way ANOVA and Dunnett's multiple comparison $p < 0.001$) (Figure 3-5B).

Progestin inhibition of DNA fragmentation

Treatment of MDA-MB-468 cells with progesterone and the selective mPR agonist Organon 02-0 resulted in a 40-55% decrease in DNA condensation detected by

TUNEL staining (Figure 3-6A). Significant decreases (Dunnett's multiple comparison *$p < 0.05$, **$p < 0.001$, $n = 15$) in DNA condensation compared to the ethanol controls were detected in the 1 and 10nM progesterone treatment groups (40 and 45% decrease, respectively) and in 10 and 100 nM Organon 02-0 treatment groups (55 and 45% decrease, respectively) but not with 10 nM R5020 treatment, indicating a non-nuclear receptor mechanism (Figure 3-6B). These results suggest that manner of cell death inhibited by progestin treatment appears to be a controlled apoptotic mechanism as opposed to an uncontrolled necrotic process.

Progestin effect on caspase 3 activity

While the mechanism of progestin inhibition of cell death appears to be regulated and apoptotic in nature, decreases in DNA fragmentation by progestins are not mediated by caspase 3 inhibition. Treatment of serum starved MDA-MB-468 cells with 1 nM-100 nM progesterone or 10nM R5020 was ineffective in altering caspase 3 activity in 5 separate experiments (one way ANOVA and Dunnett's multiple comparison $p > 0.05$, $n = 11$) (Figure 3-7).

Discussion

This study demonstrates expression and signaling of mPRs and their likely involvement in the inhibition of apoptosis in two breast cancer cell lines. We have shown that SKBR3 and MDA-MB-468 cells express three isoforms of the mPR and that the membrane progesterone binding in SKBR3 cells and progestin signaling in both cell lines display characteristics previously reported for mPRα. Progesterone-induced G protein signaling in both cell lines and progesterone decreased intracellular cAMP levels and

activated p42/44 MAP kinase in SKBR3 cells. In addition, exposure of serum-starved SKBR3 and MDA-MB-468 cells to low physiological (low nanomolar) concentrations of progesterone but not R5020, RU486, dexamethasone or estradiol-17β resulted in inhibition of cell death and DNA condensation.

mPRα, mPRβ and mPRγ mRNA and protein were found in both SKBR3 and MDA-MB-468 cells with quantitative PCR and western blot analyses yielding results similar to those found in other studies. mPRα is the predominant mPR transcript found in both cell lines and agrees with similar findings from a previous study in which the mPRα was the primary mPR transcript detected in SKBR3, MCF-7 and MDA-MB-468 breast cancer cell lines (Dressing and Thomas, 2007). Quantitative PCR analysis also demonstrated that SKBR3 cells expressed higher levels of mPRα transcript than MDA-MB-468 cells and is corroborated by western blotting for mPRα in the two cell lines. While western blotting showed a 40 kD monomer and an 80 kD dimer in both cell lines a slightly stronger mPRα dimer was seen at 80 kD in SKBR3 cells. Similarly, both quantitative PCR and western blot analyses results suggest that MDA-MB-468 cells express slightly more mPRβ than SKBR3 cells. Western blotting for mPRβ detected only a dimer of 80 kD though the mPRβ monomer has been shown previously in SKBR3 cells and in MCF7 cells (Dressing and Thomas, 2007). The detection of 40 kD and 80 kD mPRα and mPRβ bands have been ablated by primary antibody preincubation with peptide demonstrating specific antibody detection of mPRα and mPRβ monomers and dimers (Karteris et al., 2006; Thomas et al., 2007). SKBR3 cells express higher numbers of mPRγ transcript than MDA-MB-468 cells yet both cell lines express similar amounts of the 40 kD mPRγ monomer. This is the first study to examine the expression of the

mPRγ protein in breast cancer cells and these results may indicate that the relative amounts of mPRγ mRNA and protein do not directly correlate with each other. There is little published data on mPRγ expression and signaling and further studies are needed to accurately describe mPRγ expression patterns in mammalian tissues.

Western blotting also detected low levels of nPR protein in SKBR3 but not MDA-MB-468 cells. It is interesting that nPR expression is not necessary for mPR expression and corroborates findings that suggest that the mPRs are expressed and regulated in tissues independent of nPR (Karteris et al., 2006). While studies suggest that the nPR can localize to the cell membranes of cultured cells (Pedram et al., 2007), the progestin binding to SKBR3 membranes has a binding affinity for progesterone (K_D = 10.6 nM) within the range reported previously for recombinant mPRs (Thomas et al., 2007; Zhu et al., 2003a). Stronger evidence that the progestin receptor in SKBR3 cells membranes are mPRs was obtained from the steroid specificity studies. Both the membrane progesterone receptor in SKBR3 cells and recombinant human mPRα display high steroid specificity for progesterone with low affinity for testosterone and no affinity for estradiol-17β, cortisol, the nPR agonist R5020 or the nPR antagonist RU486 (Zhu et al., 2003a) (Thomas et al., 2007). Two synthetic agonists for human mPRα, Organon 02-0 and Organon 13-0, bind SKBR3 membranes with significant affinity, although with less affinity than progesterone. Taken together, these data indicate that the progesterone binding displayed by SKBR3 membranes is mediated by mPRα.

G protein activation by progesterone exposure was seen in both SKBR3 cells and MDA-MB-468 cells and is of similar magnitude to mPR activation of G proteins in other cells and tissues. The 20 to 30 percent increase in [^{35}S] GTPγ-S binding to SKBR3

membranes with 20 and 100 nM progesterone exposure is similar to the increases in [^{35}S] GTPγ-S binding to cell membranes which over-express mPRα (Thomas et al., 2007) and in human myometrial cells (Karteris et al., 2006) as well as similar to those seen in the teleost oocyte (Pace and Thomas, 2005b), sperm (Tubbs, 2007) and mammalian cells over-expressing seatrout mPRα (Thomas et al., 2007). Immunoprecipitation of the progesterone/ [^{35}S] GTPγ-S binding complex demonstrated a three fold activation of an inhibitory G protein by progesterone in SKBR3 cells which is in agreement with other studies on mPR-mediated G protein activation in female tissues. Both the identity of the G protein activated by progesterone and the level of activation is similar to that seen in human mPRα over-expressed in mammalian breast cancer cells (Thomas et al., 2007), myometrial cells (Karteris et al., 2006) and by seatrout mPRα in teleost oocytes and mammalian over-expression systems (Pace and Thomas, 2005b; Thomas et al., 2007). Progesterone consistently activates inhibitory G proteins, presumably via mPR activation, in the reproductive tissues of females, yet recent studies indicate that the mPRα is coupled to and signals via a stimulatory olfactory G protein in teleost sperm (Tubbs, 2007). It is unknown at this time whether mPR activation of inhibitory subunits is seen only in female tissues or whether mPR association with stimulatory G proteins is isolated to male gametes.

Progesterone also causes the activation of second messenger systems consistent with mPRα activation in SKBR3 cells. Exposure of SKBR3 cells to nanomolar concentrations of progesterone results in a twenty-five percent decrease in intracellular cAMP after 5 minutes. Similar decreases in intracellular cAMP upon progestin exposure are also seen in human mPRα over-expression systems (Thomas et al., 2007) as well as in

mammalian cells expressing teleost mPRα (Hanna et al., 2006; Thomas et al., 2007; Zhu et al., 2003b) and in the teleost oocyte (Pace and Thomas, 2005b). In addition to decreases in intracellular cAMP, SKBR3 cells exposed to progesterone also show a transient increase in p42/44 MAP kinase activation between 5 and 15 minutes exposure. Again, rapid activation of MAP kinases have been observed in mammalian cells over-expressing the and human myometrial cells (Karteris et al., 2006) as well as in mammalian cells expressing teleost mPRα (Hanna et al., 2006; Zhu et al., 2003b) and teleost oocytes (Pace and Thomas, 2005a). Both the progesterone binding characteristics and the rapid intracellular signaling patterns initiated by progesterone in SKBR3 cells are consistent with mPRα activation and signaling.

Treatment of SKBR3 and MDA-MB-468 with progestin inhibited serum starvation-induced apoptosis in a manner consistent with mPR mediation regardless of nPR expression. Progestins which activate the mPRs were able to inhibit serum starvation-induced apoptosis in a dose-dependent manner in both SKBR3 and MDA-MB-468 cells while R5020, RU486, dexamethasone and estradiol-17β did not. Progesterone inhibition of apoptosis in these two cell lines is similar in magnitude to studies on other breast cancer cell lines (Moore et al., 2006) as well as progesterone inhibition of apoptosis in the ovarian follicle (Engmann et al., 2006; Mussche and D'Herde, 2001; Peluso et al., 2006). Ovarian steroids have consistently been shown to regulate the proliferation and differentiation of normal mammary tissue (Feng et al., 1995; Medina, 2005; Shyamala, 1999), yet their roles in the development and progression of breast cancer are often confusing. Several reports indicate that progestins promote apoptosis in multiple breast cancer lines (Formby and Wiley, 1998; Formby and Wiley, 1999;

Kandouz et al., 1999; Kandouz et al., 1996) while others demonstrate progestin inhibition of breast cancer cell apoptosis (Bardon et al., 1987; Moore et al., 2000; Moore et al., 2006; Ory et al., 2001; Vares et al., 2004). Of particular interest is that some reports of progestin inhibition of apoptosis occur in nPR negative cell lines (Moore et al., 2006). Activation of p42/44 MAP kinase has been shown to inhibit apoptosis in several studies (Henson and Gobson, 2006; Song et al., 2005) and is a plausible mechanism for progesterone's anti-apoptotic effects in SKBR3 and MDA-MB-468 cells. Several studies have demonstrated progestin activation of growth factor signaling pathways. The nPR contains an SH3 domain which allows it to activate Src and down stream extracellular signaling-regulated kinases (ERK) (Boonyaratanakornkit et al., 2001). Other studies demonstrate a complex interplay between the nPR and ERK where the progesterone receptor rapidly activates ERK (Lange et al., 1998) and then is itself phosphorylated by ERK which alters its transcriptional activity (Daniel et al., 2007), nPR subcellular localization (Qiu et al., 2003) and degradation (Lange et al., 2000). A third set of studies suggest that the nPR interacts with the β isoform of the nuclear estrogen receptor (nERβ) (Ballare et al., 2003; Migliaccio et al., 1998) which then activates ERK and Akt through SH2 domains (Migliaccio et al., 2000). Studies examining the interaction of the nPR with growth factor signaling pathways use pico- to nanomolar concentrations of the nPR agonist R5020. Of particular note is the failure of R5020 to inhibit cell death in either cell line used in the current study. R5020 is a commonly used nPR agonist and activates the nuclear receptor but does not bind with any affinity to the mPRs (Thomas et al., 2007; Zhu et al., 2003a). The failure of R5020 to protect against death, even in an nPR positive cell line, indicates that progesterone may be acting

through a progesterone receptor other than the nPR in these cells to exert its protective effects. The activation of ERK, one of which is p42/44 MAP kinase, inhibits apoptosis via several mechanisms and phosphorylation of p42/44 MAP kinase by the mPRs has been demonstrated in multiple tissues and species. Activated MAP kinases have been shown to up-regulate the expression of anti-apoptotic members (Lin et al., 2002) and to inactivate the pro-apoptotic members (She et al., 2002) of the Bcl-2 family of proteins. In addition to phosphorylated ERK exerting anti-apoptotic signaling, activated Akt has also been shown to inhibit apoptosis both directly and indirectly. Direct effects of Akt include inhibition of BAD (Datta et al., 1997), a proapoptotic member of the Bcl-2 family, and Caspase 9 (Cardone et al., 1998), a mediator of apoptosis. Akt activation is commonly associated with the activation of βγ G protein subunits and progestins have been shown to activate Akt in the teleost oocyte which expresses mPRα (Pace and Thomas, 2005a). Thus, it is likely that Akt may also be activated in SKBR3 and MDA-MB-468 cells.

Caspase 3 activity is often regulated in association with DNA fragmentation (Porter and Jänicke, 1999) yet progesterone was not found to alter caspase 3 activity in this study. There have been recent reports of cell death or apoptosis of cancer cells in the absence of caspase activity. Serum free culture of REGb tumor cells results in cell death characterized by detached cells and DNA fragmentation in the absence of active caspase 3 or caspase 9 (Larmonier et al., 2002). A similar phenomenon is seen in cultured cortical neurons which display DNA condensation and fragmentation upon serum free culture in the absence of caspase activity (Hamabe et al., 2000). Of particular interest is that vitamin D has been shown to induce apoptotic morphology in breast cancer cells via

a mechanism that does not involve caspase activity but was blocked by the overexpression of the anti-apoptotic Bcl-2 protein (Mathiasen et al., 1999). Bcl-2 is also one of the proteins shown to be upregulated by activated ERK and Akt and may play a significant role in progesterone inhibition of apoptosis in SKBR3 and MDA-MB-468 cells. Apoptosis-like death in the absence of caspase activity has recently been recognized as an important mechanism of death in cultured cells (Blagosklonny, 2000). Some studies suggest that calpain, a calcium activated protease, and members of the Bcl-2 family of proteins may be involved in caspase-independent apoptosis (Abraham and Shaham, 2004; Borner and Monney, 1999; Mathiasen et al., 2002) and may be mediating death in serum-starved SKBR3 and MDA-MB-468 cells. Calpain, a calcium-regulated protease, is a candidate for potential mPR regulation as it has been suggested by studies in sheep that membrane progestin actions initiate calcium release, perhaps via mPR activation (Ashley et al., 2006). The Bcl-2 family of proteins also deserve further study in relation to mPR activation as various members of this family are regulated by ERK and Akt.

Conclusion

We have demonstrated endogenous mPR expression, progestin membrane binding characteristic of mPRα and rapid, progesterone-induced inhibitory G protein signaling in nPR-positive SKBR3 and nPR-negative MDA-MB-468 breast cancer cell lines. In addition, progesterone protected both cell lines from serum starvation-induced apoptosis characterized by DNA fragmentation in the absence of changes in caspase 3 activity. R5020 was not able to mirror progesterone's anti-apoptotic activity in either cell line suggesting that progesterone is likely mediating this action through mPR activation.

Further evidence that progesterone acts through the mPR to prevent apoptosis is suggested by progesterone's ability to activate ERK via mPR in the present study and Akt in previous studies. Other studies have shown that both activated ERK and Akt inhibit apoptosis through regulation of calpain and Bck-2 family proteins. Further study is needed to determine the mechanism of progesterone's protective effects, although the expression, signaling and the potential anti-apoptotic actions of the mPR in these immortalized breast cancer cell lines suggests a potential role for the mPRs in breast cancer biology.

Literature Cited

Abraham, M., and Shaham, S. (2004). Death without caspases, caspases without death. TRENDS Cell Biol *14*, 184-193.

Alkhalaf, M., El-Mowafy, A., and Karam, S. (2002). Growth inhibition of MCF-7 human breast cancer cells by progesterone is associated with cell differentiation and phosphorylation of Akt protein. Eur J Cancer Prev *11*, 481-488.

Ashley, R. L., Clay, C. M., Farmerie, T. A., Niswender, G. D., and Nett, T. M. (2006). Cloning and characterization of an ovine intracellular seven transmembrane receptor for progesterone that mediates calcium mobilization. Endocrinology *147*, 4151-4159.

Ballare, C., Uhrig, M., Bechtold, T., Sancho, E., Di Domenico, M., Migliaccio, A., Auricchio, F., and Beato, M. (2003). Two domains of the progesterone receptor interact with the estrogen receptor and are required for progesterone activation of the c-Src/Erk pathway in mammalian cells. Mol Cell Biol *23*, 1994-2008.

Bardon, S., Vignon, F., Montcourrier, P., and Rochefort, H. (1987). Steroid receptor-mediated cytotoxicity of an antiestrogen and an antiprogestin in breast cancer cells. Cancer Res *47*, 1441-1448.

Basu, A., and Rowan, B. G. (2005). Genes related to estrogen action in reproduction and breast cancer. Front Biosci *10*, 2346-2372.

Blagosklonny, M. (2000). Cell death beyond apoptosis. Leukemia *14*, 1502-1508.

Boonyaratanakornkit, V., Scott, M., Ribon, V., Sherman, L., Anderson, S., Maller, J., Miller, W., and Edwards, D. P. (2001). Progesterone receptor contains a proline-

rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. Mol Cell *8*, 269-280.

Borner, C., and Monney, L. (1999). Apoptosis without caspases: an inefficient molecular guillotine? Cell Death Diff *6*, 497-507.

Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998). Regulation of cell death protease caspase-9 by phosphorylation. Science *282*, 1318-1321.

Carnevale, R. P., Proietti, C. J., Salatino, M., Urtreger, A., Peluffo, G., Edwards, D. P., Boonyaratanakornkit, V., Charreau, E. H., Bal de Kier Joffe, E., Schillaci, R., and Elizalde, P. V. (2007). Progestin effects on breast cancer cell proliferation, proteases activation, and in vivo development of metastatic phenotype all depend on progesterone receptor capacity to activate cytoplasmic signaling pathways. Mol Endocrinol *21*, 1335-1358.

Chlebowski, R. T., Hendrix, S. L., Langer, R. D., Stefanick, M. L., Gass, M., Lane, D., Rodabough, R. J., Gilligan, M. A., Cyr, M. G., Thomson, C. A.*, et al.* (2003). Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative Randomized Trial. Jama *289*, 3243-3253.

Daniel, A., Qiu, M., Faivre, E., Ostrander, J., Skildum, A., and Lange, C. (2007). Linkage of progestin and epidermal growth factor signaling: phosphorylation of progesterone receptors mediates transcriptional hypersensitivity and increased ligand-independent breast cancer cell growth. Steroids *72*, 188-201.

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*, 231-241.

Dressing, G. E., and Thomas, P. (2007). Identification of membrane progestin receptors in human breast cancer cell lines and biopsies and their potential involvement in breast cancer. Steroids *72*, 111-115.

Engmann, L., Losel, R., Wehling, M., and Peluso, J. (2006). Progesterone regulation of human granulosa/luteal cell viability by an RU468-independent mechanism. J Clin Endo Meta *91*, 4962-4968.

Faivre, E., Skildum, A., Pierson-Mullany, L., and Lange, C. A. (2005). Integration of progesterone receptor mediated rapid signaling and nuclear actions in breast cancer cell models: role of mitogen-activated protein kinases and cell cycle regulators. Steroids *70*, 418-426.

Feng, Z., Marti, A., Jehn, B., Altermatt, H. J., Chicaiza, G., and Jaggi, R. (1995). Glucocorticoid and progesterone inhibit involution and programmed cell death in the mouse mammary gland. J Cell Biol *131*, 1095-1103.

Formby, B., and Wiley, T. S. (1998). Progesterone inhibits growth and induces apoptosis in breast cancer cells: inverse effects on Bcl-2 and p53. Ann Clin Lab Sci *28*, 360-369.

Formby, B., and Wiley, T. S. (1999). Bcl-2, survivin and variant CD44 v7-v10 are downregulated and p53 is upregulated in breast cancer cells by progesterone: inhibition of cell growth and induction of apoptosis. Mol Cell Biochem *202*, 53-61.

Fuqua, S. A., Cui, Y., Lee, A. V., Osborne, C. K., and Horwitz, K. B. (2005). Insights into the role of progesterone receptors in breast cancer. J Clin Oncol *23*, 931-932; author reply 932-933.

Hamabe, W., Fukushima, N., Yoshida, A., and Ueda, H. (2000). Serum-free induced neuronal apoptosis-like cell death is independent of caspase activity. Mol Brain Res *78*, 186-191.

Hanna, R., Pang, Y., Thomas, P., and Zhu, Y. (2006). Cell-surface expression, progestin binding, and rapid nongenomic signaling of zebrafish membrane progestin receptors alpha and beta in transfected cells. J Endocrinol *190*, 247-260.

Henson, E. S., and Gobson, S. B. (2006). Surviving cell death through epidermal growth factor (EGF) signal transduction pathways: Implications for cancer therapy. Cellular Signaling *18*, 2089-2097.

Horwitz, K. B., Zava, D. T., Thilagar, A. K., Jensen, E. M., and McGuire, W. L. (1978). Steroid receptor analyses of nine human breast cancer cell lines. Cancer Res *38*, 2434-2437.

Ikeda, K., and Inoue, S. (2004). Estrogen receptors and their downstream targets in cancer. Arch Histol Cytol *67*, 435-442.

Kandouz, M., Lombet, A., Perrot, J. Y., Jacob, D., Carvajal, S., Kazem, A., Rostene, W., Therwath, A., and Gompel, A. (1999). Proapoptotic effects of antiestrogens, progestins and androgen in breast cancer cells. J Steroid Biochem Mol Biol *69*, 463-471.

Kandouz, M., Siromachkova, M., Jacob, D., Chretien Marquet, B., Therwath, A., and Gompel, A. (1996). Antagonism between estradiol and progestin on Bcl-2 expression in breast-cancer cells. Int J Cancer *68*, 120-125.

Karteris, E., Sevasti, Z., Pang, Y., Dong, J., Hillhouse, E. W., Randeva, H. S., and Thomas, P. (2006). Progesterone signaling in human myometrium through two novel membrane g protein-coupled receptors: potential role in functional progesterone withdrawl at term. Mol Endo *20*, 1519-1534.

Lange, C., Richer, J., Shen, T., and Horwitz, K. B. (1998). Convergence of progesterone and epidermal growth factor signaling in breast cancer. J Biol Chem *273*, 31308-31316.

Lange, C., Shen, T., and Horwitz, K. B. (2000). Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. Proc Natl Acad Sci U S A *97*, 1032-1037.

Lange, C. A., Richer, J. K., and Horwitz, K. B. (1999). Hypothesis: Progesterone primes breast cancer cells for cross-talk with proliferative or antiproliferative signals. Mol Endocrinol *13*, 829-836.

Larmonier, N., Billerey, C., Rebe, C., Parcellier, A., Moutet, M., Fromentin, A., Kroemer, G., Garrido, C., Solary, E., Martin, F., and Bonnotte, B. (2002). An atypoical caspase-independent death pathway for an immunogenic cancer cell line. Oncogene *21*, 6091-6100.

Lin, H., Chen, C., Li, X., and Chen, B. D. (2002). Activation of the MEK/MAPK pathway is involved in bryostatin1-induced monocytic differenciation and up-regulation of X-linked inhibitor of apoptosis protein. Exp Cell Res *272*, 192-198.

Lyons, T. J., Villa, N. Y., Regalla, L. M., Kupchak, B. R., Vagstad, A., and Eide, D. J. (2004). Metalloregulation of yeast membrane steroid receptor homologs. Proc Natl Acad Sci U S A *101*, 5506-5511.

Mathiasen, I., Lademann, U., and Jäättelä, M. (1999). Apoptosis induced by vitamin D compounds in breast cancer cells is inhibited by Bcl-2 but does not involve known caspases or p53. Cancer Res *59*, 4848-4856.

Mathiasen, I., Sergeev, I., Bastholm, L., Elling, F., Norman, A., and Jäättelä, M. (2002). Calcium and calpain as key mediators of apopotsis-like death induced by vitamin D compounds in breast cancer cells. J Biol Chem *277*, 30738-30745.

Medina, D. (2005). Mammary developmental fate and breast cancer risk. Endocr-Relat Cancer *12*, 483-495.

Migliaccio, A., Castoria, G., Di Domenico, M., de Falco, A., Bilancio, A., Lombardi, M., Barone, M. V., Ametrano, D., Zannini, M. S., Abbondanza, C., and Auricchio, F. (2000). Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation. Embo J *19*, 5406-5417.

Migliaccio, A., Piccolo, D., Castoria, G., Di Domenico, M., Bilancio, A., Lombardi, M., Gong, W., Beato, M., and Auricchio, F. (1998). Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. Embo J *17*, 2008-2018.

Moore, M. R. (2004). A rationale for inhibiting progesterone-related pathways to combat breast cancer. Curr Cancer Drug Targets *4*, 183-189.

Moore, M. R., Conover, J. L., and Franks, K. M. (2000). Progestin effects on long-term growth, death, and Bcl-xL in breast cancer cells. Biochem Biophys Res Commun *277*, 650-654.

Moore, M. R., Spence, J. B., Kiningham, K. K., and Dillon, J. L. (2006). Progestin inhibition of cell death in human breast cancer cell lines. J Steroid Biochem Mol Biol *98*, 218-227.

Mussche, S., and D'Herde, K. (2001). Contribution of progesterone, follicle stimulating hormone and glucocorticoids in survival of serum-free cultured granulosa cell explants. J Endo *169*, 321-331.

Ory, K., Lebeau, J., Levalois, C., Bishay, K., Fouchet, P., Allemand, I., Therwath, A., and Chevillard, S. (2001). Apoptosis inhibition mediated by medroxyprogesterone acetate treatment of breast cancer cell lines. Breast Cancer Res Treat *68*, 187-198.

Pace, M., and Thomas, P. (2005a). Steroid-induced oocyte maturation in Atlantic croaker (*Micropogonias undulatus*) is dependent on activation of the phosphatidylinositol 3-kinase/Akt signal transduction pathway. Bio Reprod *73*, 988-996.

Pace, M. C., and Thomas, P. (2005b). Activation of a pertussis toxin-sensitive, inhibitory G-protein is necessary for steroid-mediated oocyte maturation in spotted seatrout. Dev Bio *285*, 70-79.

Pedram, A., Razandi, M., Sainson, R., Kim, J., Hughes, C., and Levin, E. (2007). A conserved mechanism for steroid receptor translocation to the plasma membrane. J Biol Chem *282*, 22278-22288.

Peluso, J., Pappalardo, A., Losel, R., and Wehling, M. (2006). Progesterone membrane receptor component 1 expression in the immature rat ovary and its role in mediating progesterone's antiapoptotic action. Endocrinology *147*, 3133-3140.

Porter, A., and Jänicke, R. (1999). Emerging roles of caspase-3 in apoptosis. Cell Death Diff *6*, 99-104.

Qiu, M., Olson, A., Faivre, E., Horwitz, K. B., and Lange, C. (2003). Mitogen-activated protein kinase regulates nuclear association of human progesterone receptors. Mol Endocrinol *17*, 628-642.

She, Q. B., Ma, W. Y., Zhong, S., and Dong, Z. (2002). Activation of JNK1, RSK2, and MSK1 is involved in serine 112 phosphorylation of Bad by ultraviolet B radiation. J Biol Chem *277*, 24039-24048.

Shyamala, G. (1999). Progesterone signaling and mammary gland morphogenesis. J Mammary Gland Biol *4*, 89-104.

Skildum, A., Faivre, E., and Lange, C. A. (2005). Progesterone receptors induce cell cycle progression via activation of mitogen-activated protein kinases. Mol Endocrinol *19*, 327-339.

Song, G., Ouyang, G., and Bao, S. (2005). The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med *9*, 59-71.

Tang, Y. T., Hu, T., Arterburn, M., Boyle, B., Bright, J. M., Emtage, P. C., and Funk, W. D. (2005). PAQR proteins: a novel membrane receptor family defined by an ancient 7-transmembrane pass motif. J Mol Evol *61*, 372-380.

Thomas, P., Pang, Y., Dong, J., Groenen, P., Kelder, J., de Vlieg, J., Zhu, Y., and Tubbs, C. (2007). Steroid and G protein binding characteristics of the seatrout and human

progestin membrane receptor alpha subtypes and their evolutionary origins. Endocrinology *148*, 705-718.

Tubbs, C. (2007) Mechanisms of progestin–stimulated sperm hypermotility in two teleosts: the Atlantic croaker (*Micropogonias undulatus*) and the southern flounder (*Platylicthys lethigstomata*), Ph. D., The University of Texas, Port Aransas.

Vallejo, G., Ballare, C., Baranao, J. L., Beato, M., and Saragueta, P. (2005). Progestin activation of nongenomic pathways via cross talk of progesterone receptor with estrogen receptor beta induces proliferation of endometrial stromal cells. Mol Endocrinol *19*, 3023-3037.

Vares, G., Ory, K., Lectard, B., Levalois, C., Altmeyer-Morel, S., Chevillard, S., and Lebeau, J. (2004). Progesterone prevents radiation-induced apoptosis in breast cancer cells. Oncogene *23*, 4603-4613.

Zhu, Y., Bond, J., and Thomas, P. (2003a). Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. Proc Natl Acad Sci U S A *100*, 2237-2242.

Zhu, Y., Rice, C. D., Pang, Y., Pace, M., and Thomas, P. (2003b). Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc Natl Acad Sci U S A *100*, 2231-2236.

Table 3-1: Primer sequences used for QPCR on breast cancer cell lines.

Protein Name	Sequence: Sense Anti-sense	Fragment Size (base pairs)
mPRα	5'cgctcttctggaagccgtacatctatg 5'cagcaggtgggtccagacattcac	100
mPRβ	5'agcctcctacatagatgctgccc 5'ggtgcctggttcacatgttcttca	194
mPRγ	5'cagctgtttcacgtgtgtgtgatcctg 5'gcacagaagtatggctccagctatctgag	120
B-actin	5'gcgggaaatcgtgcgtgacatt 5'gatggagttgaaggtagtttcgtg	305

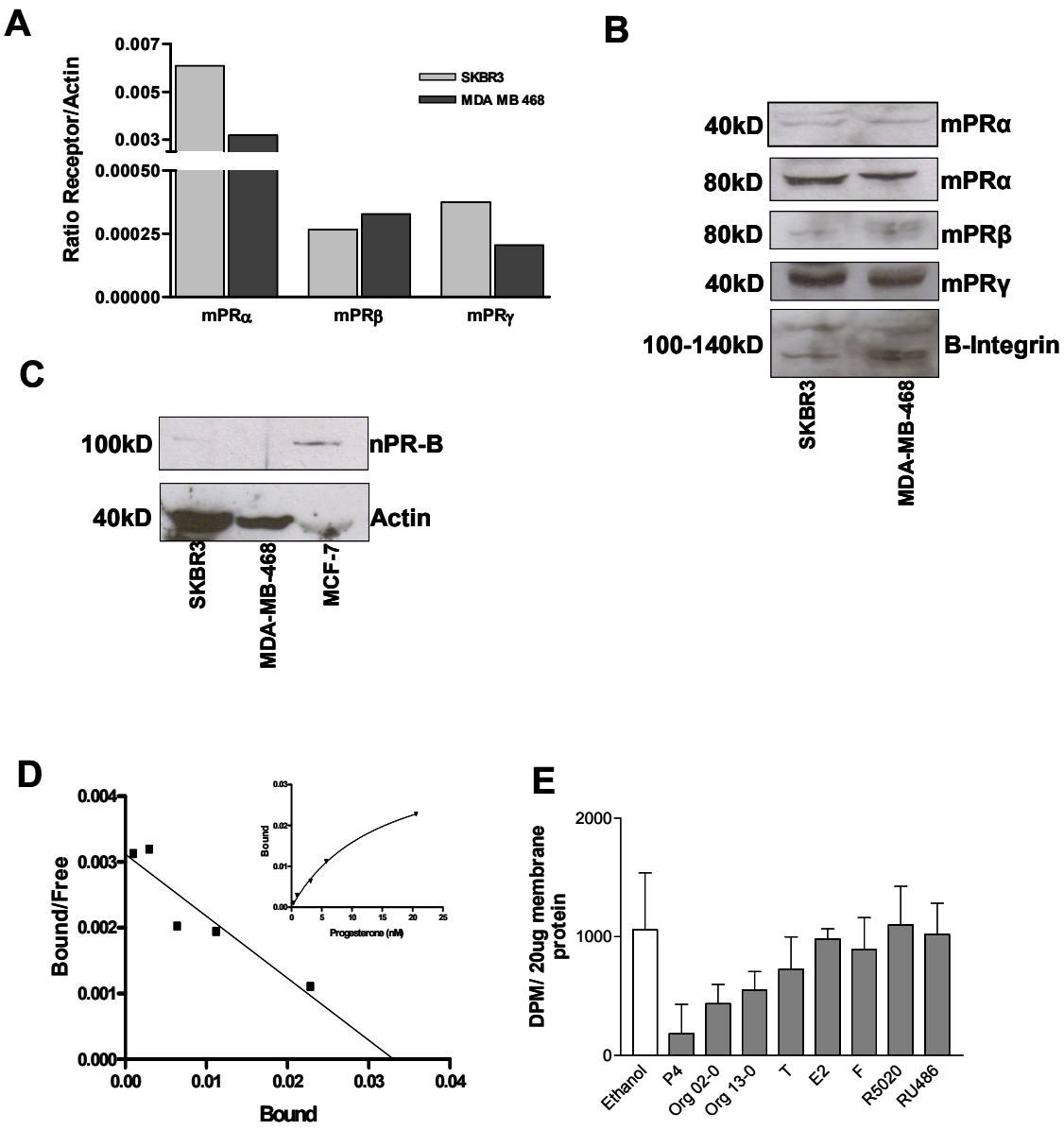

Figure 3-1: Progesterone receptor expression in and progesterone binding to cancer cell membranes. A. Quantitiative RT-PCR on RNA isolated from SKBR3 and MDA-MB-468 cells using primers directed against mPRα, mPRβ and mPRγ. B. Western blotting for mPRα, mPRβ and mPRγ on SKBR3 and MDA-MB-468 cell membranes. C. Western blotting for nPR in SKBR3 and MDA-MB-468 cell lysates. MCF-7 lysate used as positive control. D. [3H] progesterone saturation binding on SKBR3 plasma membranes. Kd 10.59nM Bmax: 0.33nmol/mg protein. E. Single point competition of 50nM steroid for SKBR3 membrane binding against 2nM [3H] progesterone.

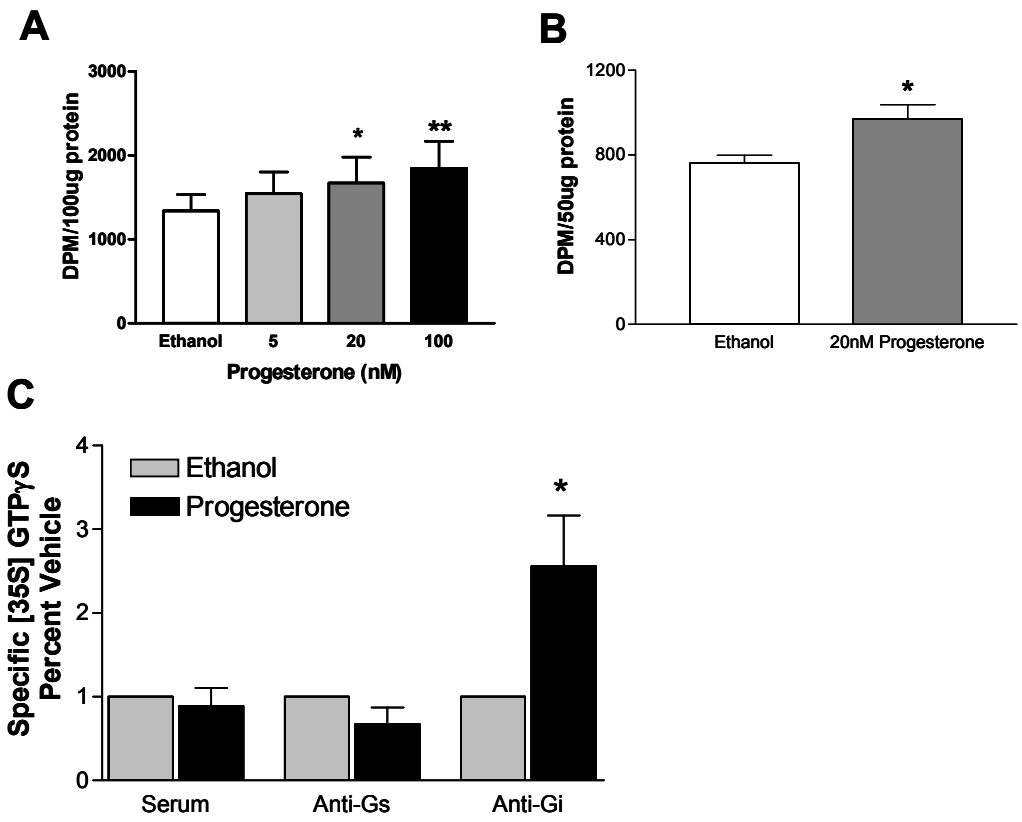

Figure 3-2: G protein activation in response to progesterone. **A**. Specific binding of [35S] GTPγS to SK-BR-3 membrane fractions in response to increasing concentrations of progesterone. Data represent mean ± SEM. Statistically significant differences from ethanol control were determined using one-way paired ANOVA, and Dunnett's Multiple Comparison *p<0.05, **p<0.001, n=4. B. Specific binding of [35S] GTPγS to MDA-MB-468 cell membranes. Data represent means ± SEM. Significant differences from ethanol were determined using Student's t-test *p<0.05, n=3. **C**. Immunoprecipitation of [35S] GTPγS in response to 20-100nM progesterone by G protein antibodies in SK-Br-3 cell membranes. Data represent mean percent ethanol specific [35]GTPγS binding. Differences from control determined by one way *ANOVA and Dunnett'smulitple comparison p<0.05, n=5.

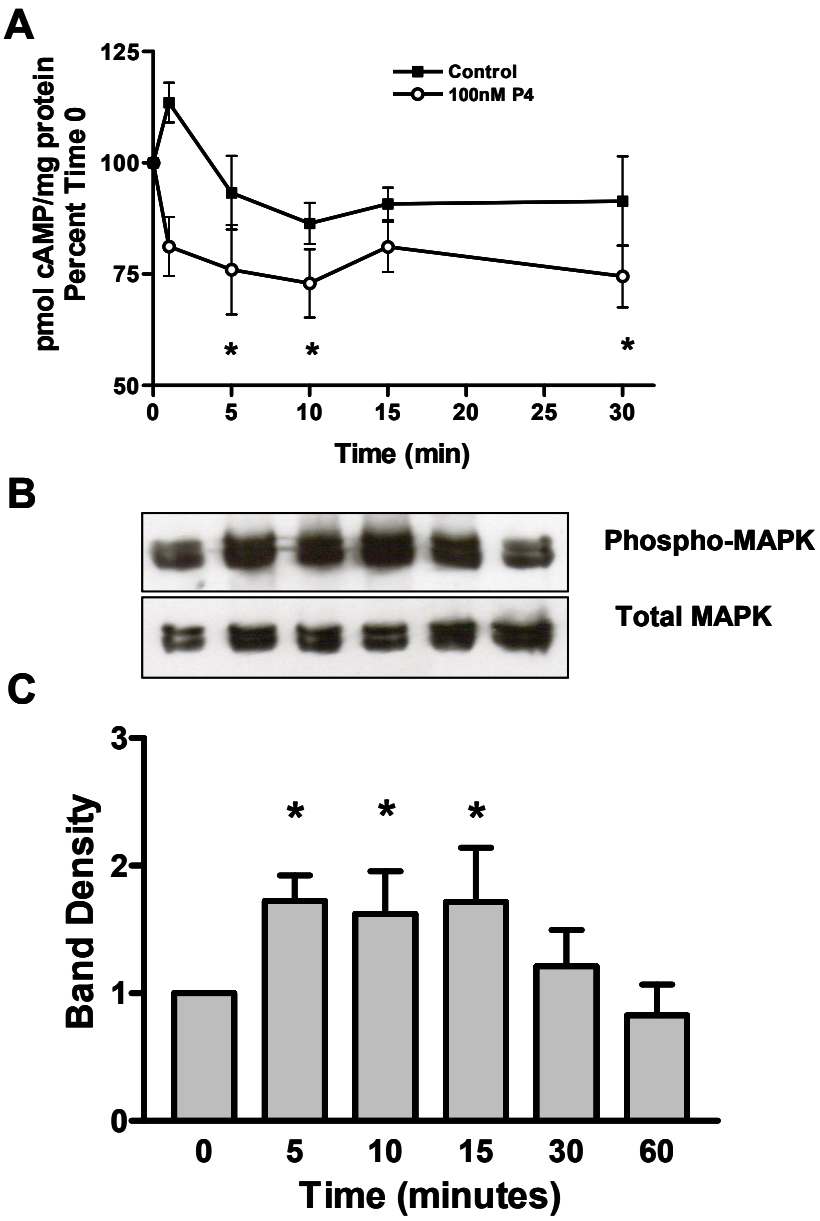

Figure 3-3: Intracellular signaling in response to progesterone exposure. A. cAMP concentration time course in whole SK-BR-3 cells in response to 100nM progesterone. *Data represents mean percent time 0 cAMP normalized to protein ± SEM. Statistical difference from time 0 determined by paired one way ANOVA and Dunnett's multiple comparison $p<0.05$, n=5. B.C. MAP kinase activation by 100nM progesterone in SK-BR-3 cells. Representative blot (B) and densitometry (C) from four separate experiments shown. Data represent mean phospho-p42/44 band density normalized to total p42/44 band density ± SEM. Statistically significant differences from time 0 were determined using paired one way ANOVA and Dunnett's multiple comparison *$p<0.05$, n=4.

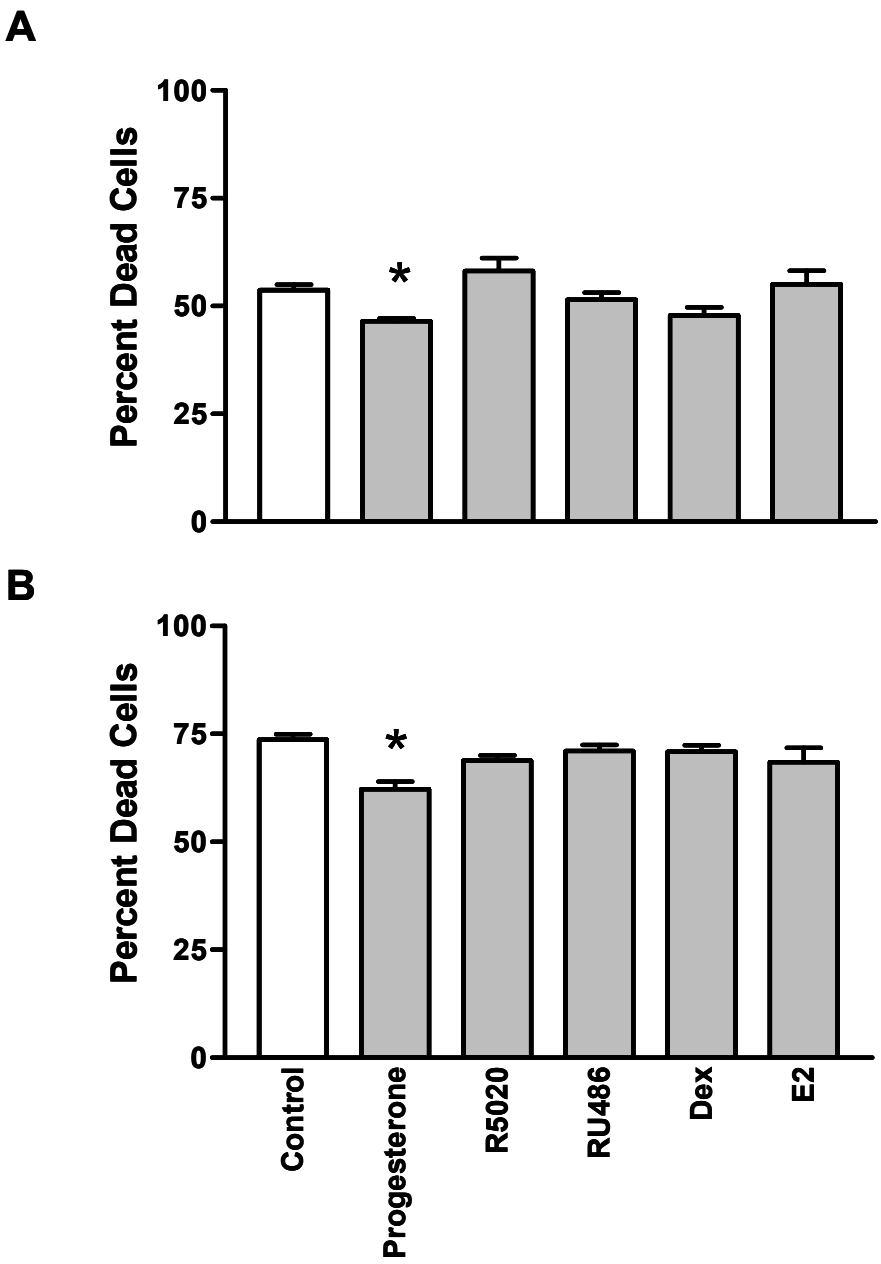

Figure 3-4: Alteration of serum starvation-induced cell death by 10nM steroid. A.. SK-BR-3 cell death determined by trypan blue exclusion after 5-7 days of serum starvation. Data represent mean ± SEM. Difference between ethanol and steroid treatment determined by one way ANOVA and Dunnett's multiple comparison on square root transformed data *$p<0.05$, n=6. B. MDA-MB-468 cells. Data represent mean ± SEM. Significant difference between ethanol and steroid treatment determined by one way ANOVA and Dunnett's multiple comparison *$p<0.01$, n=6.

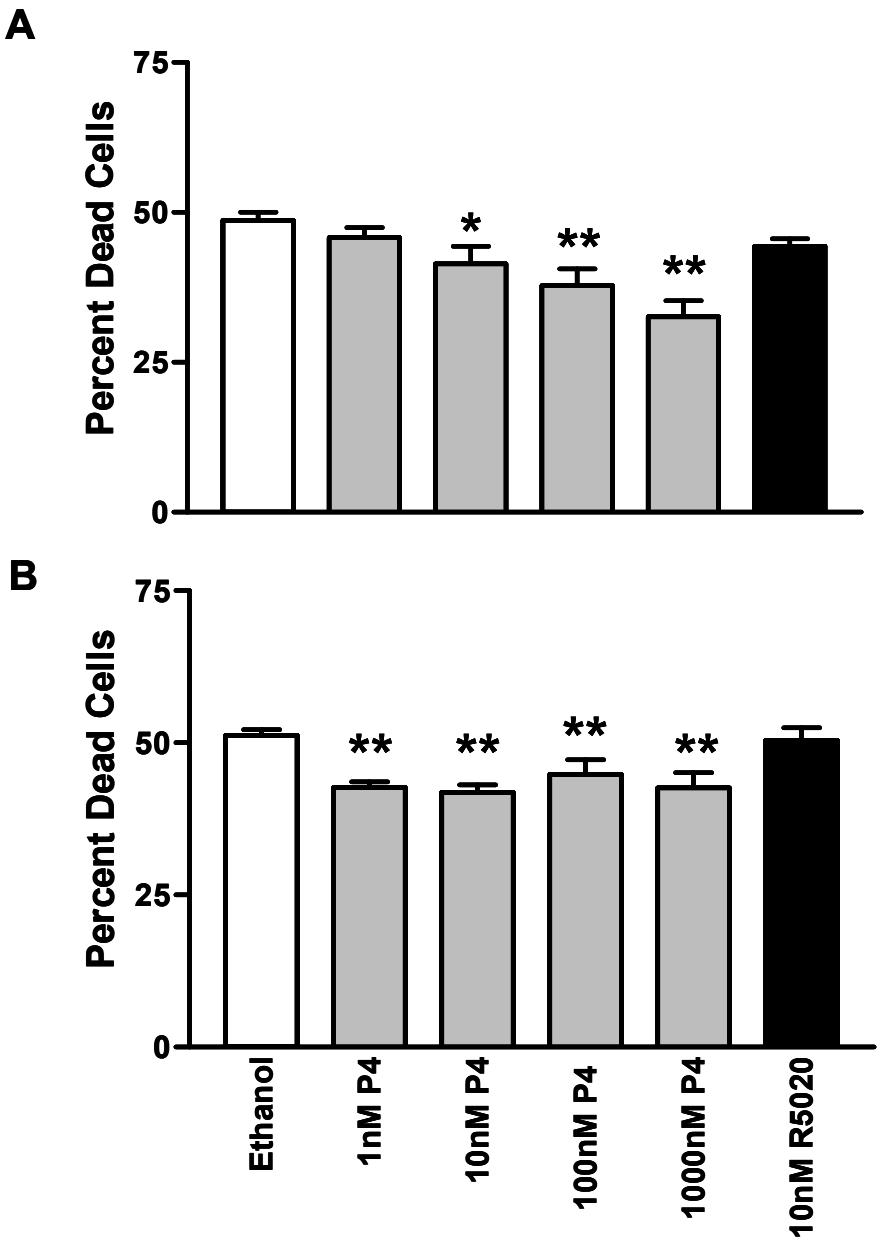

Figure 3-5: Cell death in response to progestins. A. SKBR3 cell death determined by trypan blue exclusion in response to progesterone after 5 days of serum starvation. Data represent mean death ± SEM. Significant difference from control determined by one way paired ANOVA and Dunnett's multiple comparison *p<0.05 **p<0.001, n=9. B. MDA-MB-468 cell death after 4 days exposure to progesterone. Data represent mean death ± SEM. Significant difference from ethanol determined by one way paired ANOVA and Dunnett's multiple comparison **p<0.001, n=6.

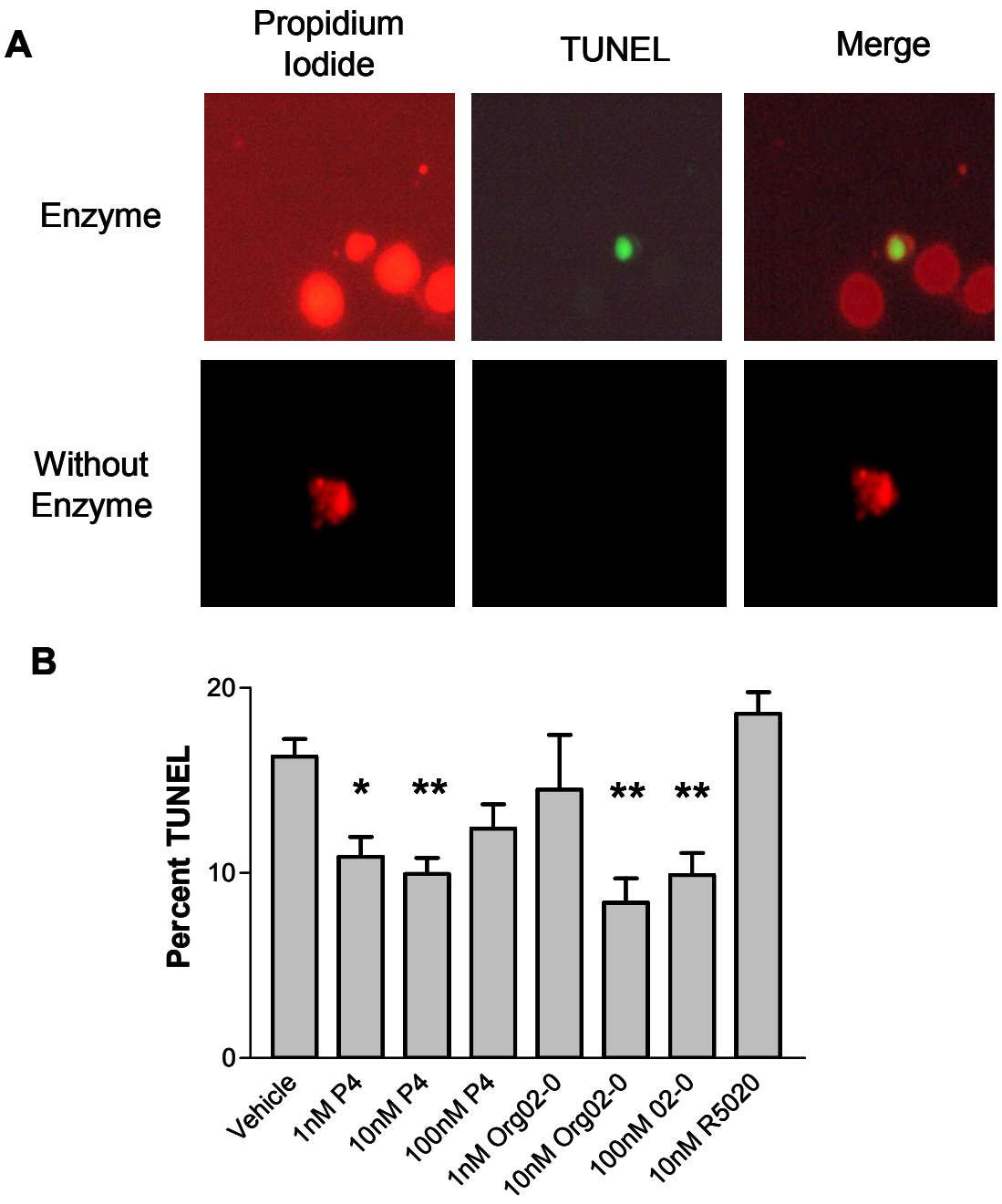

Figure 3-6: DNA fragmentation in MDA-MB-468 cells in response to progestins. A . TUNEL staining B. TUNEL staining in response to 48 hours exposure to progestins. Data represent mean percent TUNEL stained nuclei ± SEM. Differences between vehicle and treatment determined by one way ANOVA and Dunnett's multiple comparison *p<0.05, **p<0.001, n=15.

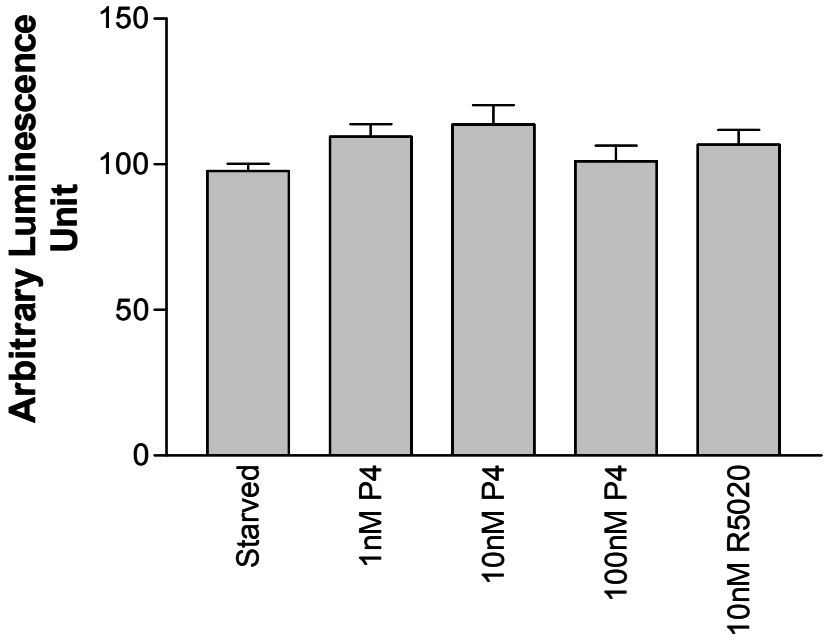

Figure 3-7: Progesterone effect on caspase 3 activity. Effect of 10nM progesterone on caspase 3 activity in MDA-MB-468 cells. Data represent mean activity normalized to protein and control treatments. Difference from control determined by one way ANOVA and Dunnett's mutiple comparison n=11.

Chapter 4

Membrane progesterone receptor α, β and γ expression in paired normal and malignant human breast biopsies and their expression patterns in relation to other steroid receptors

Summary

The expression and signaling of nuclear steroid hormone receptors in human breast cancer is a topic of intense research and has been used as a diagnostic tool in breast cancer treatment for nearly 40 years. Several laboratory and clinical studies in the last 20 years have suggested that progesterone plays a significant role in breast cancer development, progression and metastasis independent of its modulation of estrogen effects. While there is information concerning progesterone's activity in breast cancer via the nuclear progesterone receptor (nPR), there are reports of progesterone actions in breast cancer cells which do not express the nPR. Recently three isoforms of a recently-discovered novel membrane localized progesterone receptor (mPRα, mPRβ and mPRγ) were identified in several human breast cancer cell lines and therefore are candidates as the receptors involved in progesterone's anti-apoptotic effects in breast cancer. Quantitative reverse-transcription PCR was used in the current study to examine the expression patterns of the three mPR isoforms in paired normal and malignant breast biopsies from 13 women. While expression of the mPRs was lower than that of the nuclear steroid receptors in all of the biopsy samples examined, one or more of the mPR isoforms was upregulated in 85% and mPRγ was upregulated in 70% of the tumors

examined. Further analysis indicated that mPRα upregulation appeared to be associated with nuclear progesterone receptor upregulation and mPRγ upregulation was linearly correlated to the breast epithelial cell marker CK19. This study is the first to examine mPR expression levels in normal and malignant human breast biopsies. The patterns of mPR expression observed in malignant breast tissue in the present study suggest a potential role for the mPRs in breast cancer biology.

Introduction

Steroid receptor-positive tumors constitute up to seventy percent of the diagnosed breast cancer cases in the United States (American Cancer Society). Steroid receptor status influences patient treatment and may serve as an indicator of survival. Investigations of steroid receptor influence on breast cancer development, progression and treatment has primarily focused on nuclear estrogen receptor (ER) function. Most treatment options directed against steroid responsive cancers inhibit estrogen promotion of cell growth and division by using selective estrogen receptor modulators (SERMs) to block or impair the ability of estrogen to activate the ER and/or aromatase inhibitors which inhibit endogenous estrogen production. SERMs used to treat ER-positive breast tumors include the popular drugs tamoxifen and raloxifene and aromatase inhibitors include arimidex, letrozole and exemestane.

The presence of the nuclear progesterone receptor (nPR) in breast tumors has often been viewed as an indicator of a functional estrogen receptor (Olivotto et al., 2004; Osborne et al., 1980) as nPR expression can be regulated by estrogen (Nardulli et al., 1988). Yet several studies now suggest that this may be an oversimplification of

progesterone and nPR action in breast cancer (Fuqua et al., 2005; Moore, 2004). Indeed multiple lines of research now suggest that progesterone may have a role in breast cancer development and progression independent of estrogen. Multiple immortalized human breast cancer cell lines express the nPR in the absence of the ER (Horwitz et al., 1978) and there are clinical reports of nPR-positive ER-negative tumors (Dunnwald et al., 2007; Keshgegian, 1994; Keshgegian and Cnaan, 1996). Studies in ER-negative nPR-positive cell lines show that progestin influences growth and cell cycle progression via a mechanism involving the ability of the nPR to activate cytoplasmic signaling pathways (Boonyaratanakornkit et al., 2001; Carnevale et al., 2007; Faivre et al., 2005; Lange et al., 1999; Lange et al., 1998; Richer et al., 1998; Skildum et al., 2005). Progestins have also been shown to promote cell survival and inhibit apoptosis in nPR-negative cell lines (Moore et al., 2006), suggesting novel progesterone receptors may have important roles in breast cancer biology.

Clinical evidence for a direct influence of progestins, in addition to the well known effects of estrogens, on breast cancer development and growth has been demonstrated in several clinical trials, most notably the controversial early conclusion of the Women's Health Initiative estrogen plus progestin hormone therapy due to an increase in the incidence in breast tumor development (Chlebowski et al., 2003; Li et al., 2000; Persson et al., 1999; Ross et al., 2000; Schairer et al., 2000). Thus, there is growing awareness of the importance of progesterone and progesterone receptors in breast cancer biology and as a result progesterone's effects on breast cancer cell lines and in breast tumors are becoming more widely studied.

Progesterone inhibition of serum starvation- induced apoptosis on two immortalized breast cancer cell lines, one which expressed the nPR and one which did not, was examined earlier in this dissertation (Chapter 3). The mediator of this progesterone action is likely the novel membrane progesterone receptor (mPR) which suggests an important role for the mPRs in breast cancer biology. The mPR is a novel gene, unrelated to the nPR, first identified in the ovary of a teleost fish and three isoforms (mPRα, mPRβ and mPRγ) were subsequently cloned in humans (Zhu et al., 2003a; Zhu et al., 2003b). The mPRs are expressed in all immortalized breast cancer cell lines examined to date although the ratio of each isoform differs between cell lines (unpublished observation).

The goal of the current study is to examine the expression patterns of mPRs in relation to other ovarian steroid receptors and malignancy. The current study examines the expression of the three human mPR isoforms, the nPR, two ERs, the putative progestin receptor PGRMC1and CK19, a breast epithelial cell marker, in paired normal and malignant human breast biopsies from thirteen women.

Methods

Tissue Samples

Paired normal and malignant biopsy samples were obtained from the NCI Human Tissue Network. Samples were handled in accordance with NIH guidelines approved by The University of Texas office of Research Support and Compliance. Samples were stored at -80^{o}C until use. Descriptions for each of the samples is provided in Table 4-2.

RNA Isolation

RNA was isolated from 2-3 mg tissue samples in Tri Reagent (Sigma St. Louis MO) as per the manufacturer's instructions. Following RNA isolation, samples were DNase treated using the DNA free RNA kit from Zymo Research (Orange CA). DNA contamination was tested in the absence of reverse transcriptase using primers directed against β-actin. Samples with DNA contamination were DNase treated a second time.

QRT-PCR

QRT-PCR was performed on approximately 250 μg DNA-free RNA using Brilliant II SYBR Green QRT-PCR Mastermix 1-Step (Stratagene, Cedar Creek TX) on an Eppendorff RealPlex ep^2 (Hamburg, Germany) for mPRα, mPRβ, mPRγ, nPR, ERα, ERβ, PGRMC1, CK19 and β-actin (See Table 4-1 for primer sequences). Primers were used at final concentrations of 100 nM. The cycling protocol used following a 30 minute 50°C reverse transcription incubation time and a 10 minute 95°C denaturation incubation was as follows: 30 seconds at 95°C, 60 seconds at 55°C and 30 seconds at 72°C for 40 cycles. ERα and ERβ primers were purchased from RealTimePrimers. CK19 primers were developed by Stathopoulou et al (2003), PGRMC1 by Crudden et al (2005) and NPR by Latil et al (2001). No template control samples were performed for each sample to confirm the specificity of the reaction (data not shown).

Determination of Relative Receptor Expression

CT values were calculated using Eppendorf software (Hamburg, Germany) and receptor concentrations were normalized to β-actin expression taking into account primer efficiency using the method developed by Fink (Fink et al., 1998).

Results

mPR expression levels relative to other receptors

mPRα mRNA was 2-3 times more abundant in both normal and malignant tissues than either mPRβ or mPRγ suggesting that mPRα is the primary mPR expressed in breast tissue. Yet overall, the expression levels of the mPRs are 2-5 times lower than other ovarian steroid receptors measured (Figure 4-1).

mPR expression relative to malignancy

Expression of mPRα, mPRβ and mPRγ mRNAs were detected in all 26 breast tissue biopsies examined. Average mPRα, mPRβ and mPRγ mRNA levels are slightly upregulated in malignant breast tissue over normal tissue from the same breast (Figure 4-1). Ratios of malignant to normal gene expression ranged from 0.01 to over 100. In order to simplify the data here we define upregulation as greater than 1.2:1 ratio of expression in malignant tissue to normal tissue which corresponds to a 20% increase in gene transcription (Table 4-3). Of the thirteen women examined, mPRα was upregulated in 6 cases (46%), mPRβ was upregulated in 5 (38%) and mPRγ in 9 (70%, Table 4-3). Overall mPRγ was upregulated in a higher percentage of patients with malignant breast tissue (70%), than any of the other receptors (nPR: 54%; ERα: 46%; ERβ: 38%; PGMRC1: 38%)

mPR upregulation in malignant tissue relative to other genes

Upon examination of the profile of gene upregulation in each individual, several patterns emerged with regard to receptor co-regulation (Table 4-3). Only three cases (biopsy nos. 4, 7, 13) showed no upregulation of any mPR isoform. None of the other receptors were upregulated either in these three cases, indicating an overall absence of steroid receptor regulation in these biopsy samples. Of the remaining ten biopsy samples,

nine showed upregulation of mPRγ. Three cases had only one mPR isoform upregulated in malignant tissue and two mPR isoforms were upregulated in 4 cases (biopsy nos. 2, 3, 6, 12). Of particular interest is that in three cases all three mPR isoforms are upregulated (biopsy nos.1, 5, 8). In all 6 cases where mPRα and most cases where mPRβ are upregulated mPRγ was also upregulated. All six of the women who displayed upregulated mPRα expression in malignant tissue also displayed upregulation of the nPR. Similarly, seven out of the nine biopsies showing upregulation of mPRγ also displayed upregulation of nPR. Although four out of five biopsies with upregulation of mPRβ expression also displayed upregulation of nPR, three other biopsies with increased nPR expression showed no increase in mPRβ. The pattern of upregulation of the other putative progesterone receptor, PGMRC1, differed from those of the other receptors. Half of the biopsies showing upregulation of the mPRs (5/10) and five out of seven biopsies displaying upregulation of nPR did not have parallel increases in PGMRC1 expression. The parallel regulation of mPRα, mPRγ and nPR in breast cancer tissues suggests the possibility of an interaction between progesterone receptors, perhaps with important implications in breast cancer biology. There was a linear relationship between CK19 and mPRγ upregulation. Linear regression determined a positive relationship between nPR and mPRγ with a slope of 14.42 ± 3.846. $p < 0.005$ indicated a slope significantly different from 0 with $r^2 = 0.5609$ (Figure 4-2A). Linear regression was used to determine a positive relationship between CK19 and mPRγ with a slope of 0.1430 ± 0.02262. $p < 0.0001$ indicated a slope significantly different from 0 with $r^2 = 0.7842$ (Figure 4-2B). The expression pattern of ERβ upregulation differed from those of the other receptors, including ERα, and was only observed in two of the thirteen cases. In

contrast, upregulation of ERα corresponded to upregulation of mPRα in four out of six cases (67%) and nPR the same number of times (67%). There was no correlation between mPR expression and tumor origin, race or Bloom/Richardson Grade. Alternatively age does appear to influence mPR expression patterns. The average age of women who show no mPR upregulation or only mPRγ upregulation was 41 years old while the average age of women who showed upregulation of two or more mPR isoforms was 59, suggesting that mPR upregulation may occur in breast cancer of postmenopausal women more frequently than in younger women.

Discussion

The current study is the first to describe the expression patterns of mPR isoforms in human breast cancer biopsies. While the transcript levels of mPRs are somewhat lower compared to nuclear steroid receptors, mPRγ appears to be upregulated in 70 percent of the tumors examined which is similar to the proportion of tumors defined as steroid receptor positive (Varghese, 2007). The ratio of mPRγ upregulation in malignant breast tissue is also positively correlated with CK19 upregulation, a marker of epithelial cells. We also discovered that mPRα, the most abundant of the mPR isoforms, and mPRγ, the most ubiquitous of the mPR isoforms, appear to be co-regulated with nPR which suggests the possibility of an interaction between the two progesterone receptors.

This study shows that the mPRs are widely expressed in breast tumors with at least one mPR isoform upregulated in 85% of the tumors examined. Steroid receptor expression in breast cancer has been used for 30 years to determine tumor responsiveness to endocrine therapy (Allegra et al., 1980; De Sombre et al., 1974; Horwitz and McGuire,

1975; Knight et al., 1980) and future prognosis. Several studies suggest that patients with steroid responsive tumors have lower mortality rates than ER-/PR- cancers (Aaltomaa et al., 1991; Anderson et al., 2001; Crowe et al., 1991; Dunnwald et al., 2007; Fisher et al., 1988; Parl et al., 1984; von Maillot et al., 1982) (Furmaniski et al., 1980; Howat et al., 1985). This is likely due to the finding that the degree of tumor differentiation and histology grade is the single most important histological risk factor for survival in breast cancer (Parl and Dupont, 1982) and steroid responsive tumors, in general, show a higher degree of differentiation (Reiner et al., 1988).

Breast tumor steroid responsiveness has historically focused on estrogen but several studies have begun to examine the effects of progesterone on breast tumor development, growth and metastasis. While most steroid receptor-expressing tumors express the ER in addition to the nPR, there are reported cases of ER-/nPR+ tumors. Patients with ER-/nPR+ tumors have a poorer clinical outcome than patients with ER+/nPR+ tumors with a significantly higher incidence of tumor recurrence and a three times higher death rate (Keshgegian and Cnaan, 1996). Though a role for progesterone in breast cancer is difficult to define (Lange, 2007), the results in the previous chapter are in agreement with those of recent studies showing that progestins act to promote immortalized breast cancer cell growth (Boonyaratanakornkit et al., 2007; Carnevale et al., 2007; McGowan et al., 2007; Migliaccio et al., 1998; Skildum et al., 2005) and inhibit cancer cell apoptosis (Moore et al., 2000; Moore et al., 2006; Ory et al., 2001; Vares et al., 2004). These actions of progesterone often occur via nPR activity but progesterone has been shown to inhibit apoptosis in the absence of the nuclear progesterone receptor,

as demonstrated in Chapter 3, indicating that progesterone may be acting through an alternative progesterone receptor to maintain cancer cells.

This study uses quantatitative reverse-transcription PCR to determine the levels of steroid receptor expression. The likely responsiveness of tumors to steroids was originally determined via biochemical characterization of nuclear receptors in biopsied tissue. Ligand binding using radiolabeled estrogen receptor agonists and progesterone receptor agonists was used to determine ER/nPR concentration where a minimal steroid binding level (mol steroid/mg protein) was established to determine steroid receptor positive or negative status (Geyer et al., 1985). Recently, immunohistochemical detection of ER and nPR has replaced biochemical characterization to determine tumor steroid receptor status. Breast tumors are known to express at least two forms of the ER (α and β) as well three forms of the nPR (the full length nPR-B and two truncated forms nPR-A and nPR-C). In addition, several studies demonstrate the presence of novel steroid receptors in breast tumors including the estrogen binding GPR30 (Filardo et al., 2007; Filardo and Thomas, 2005; Thomas et al., 2005) and the current study demonstrates mPRα, β and γ expression in breast tumors, all of which have been shown to bind progesterone in other expression systems and tissues (Karteris et al., 2006; Thomas et al., 2007; Zhu et al., 2003a). Biochemical characterization of tumor steroid receptor status does not yield information on the identity of the receptor binding the steroid. Immunohistological testing of breast biopsies is able to yield information on the receptor types expressed in the tumor and also the tissue localization of the receptors and is currently the favored method. Yet it is often difficult to determine receptor concentration between samples and between receptors in immunohistological diagnostics

due to differences in antibody strength. Thus, studies, including this one, have begun to use quantitative PCR to examine the identities and levels of steroid receptors expressed in tumors (De Bessa et al., 2006; Latil et al., 2001; Perou et al., 2000; Sorlie et al., 2001). Quantitative PCR has the advantages of being able to examine receptor expression in very small tissue or cell samples as well as yielding data on the levels of specific steroid receptors present relative to each other. In addition, the development and characterization of primers directed against genes for quantitative PCR takes a relatively short period of time and yields a higher level of specificity in comparison to generation and characterization of antibodies directed against proteins. The most problematic disadvantage of using PCR to examine gene expression is that it does not measure protein levels. Additionally, biopsies contain variable amounts of receptors in neoplastic tissue compared to non proliferative tissue and while laser assisted micro-dissection now allows for the selection of a specific cell type for PCR analysis immunohistochemistry is often the method of choice in determining the pattern of receptor expression between different cell types in and around the breast tumor.

In the present study mPRγ was upregulated in the majority of tumors in which any mPR isoform was upregulated but the upregulation of mPRα and mPRβ demonstrated patterns different from mPRγ and from each other. mPRα and mPRβ were upregulated in 45% and 40% of the tumors examined respectively. Of the 60% of tumors in which either mPRα or mPRβ were upregulated only 23% showed upregulation of both isoforms. The differences in individual tumors between mPRα and mPRβ upregulation suggest that mPR isoform expression patterns may be important in breast cancer biology. The patterns of steroid receptor isoform expression often differ between normal and

malignant breast and have been shown to affect the clinical response to treatment and prognosis. Studies examining the expression patterns of ERα and ERβ show unrelated differential expression patterns of the two receptors in malignant breast tissue and suggest that ERβ is downregulated in malignant breast tissue (Shaw et al., 2002). Other studies show that tumors which express high levels ERβ in addition to ERα respond more favorably to endocrine therapy (Hopp et al., 2004b; Lin et al., 2007) and suggest that the patterns of ERα and ERβ expression relative to each other may influence the expression of estrogen responsive cell proliferation genes (Shupnik, 2007). The ratios of progesterone receptors have also been shown to influence response to treatment and prognosis. The ratios of nPR-B and the truncated nPR-A are associated with the histological grade of the tumor (Ariga et al., 2001) and are suggested to influence the response to tamoxifen where patients with a higher ratio of nPR-A to nPR-B have lower disease-free survival rates than those with a low ratio (Hopp et al., 2004a). While there is currently no data on the expression patterns of the mPRs in relation to treatment response or prognosis, other studies suggest that the ratios of steroid receptor isoforms influence both of these factors and suggest that further study of mPR ratios in relation to treatment and prognosis is warranted.

Cytokeratin19 (CK19) expression was measured in the current study and its upregulation in malignant breast tissue was found to be linearly correlated with mPRγ upregulation. Regression analysis between CK19 and mPRγ malignant to normal ratios indicated that expression levels of the two are positively correlated. CK19 is a commonly used breast epithelial cell marker. It is most often used as a diagnostic tool to examine circulating epithelial cell levels of breast origination and indicates lymph node

metastasis (Iakovlev et al., 2008; Ji et al., 2006; Visser et al., 2008). CK19 is normally heterogeneously expressed in breast tissue with expression in lumnal cells, although its expression appears to be upregulated and homogeneous in breast tumors (Su et al., 1996). CK19 also appears to be an important prognostic tool in tumors. A lack of CK19 expression in tumors was associated with high rates of relapse both at the tumor site and distantly as well as decreased survival rates (Parikh et al., 2008). Although this study does not directly examine mPR upregulation in relation to prognosis, the positive correlation between CK19 expression and mPRγ may indicate a favorable prognosis for patients with mPR expressing tumors.

There was no upregulation of any steroid receptor, including the mPRs, in three of the thirteen tumors examined in this study. One of the three tumors is classified as a medullary carcinoma while the others are characterized as infiltrative ductal carcinoma. Medullary carcinomas are often characterized as having low CK19 expression, being mostly ER-/PR- and often Her-2/neu positive (Jensen et al., 1996; Larsimont et al., 1994; Xu et al., 2003). The Her-2/neu gene encodes epidermal growth factor receptors and is upregulated in around 25% of breast cancers (Slamon et al., 1987; Slamon et al., 1989). Although much remains unknown concerning the interaction between steroid responsiveness and Her-2/neu over-expression in tumors, several studies suggest a link between the two. Multiple studies suggest that Her-2/neu over-expression may be associated with tamoxifen resistance in both steroid responsive and unresponsive tumors (Konecny et al., 2003; Nicholson et al., 1990; Peiro et al., 2007; Wright et al., 1992). While Her-2/neu was not measured in this study, and no direct associations between the mPRs and Her-2/neu can be made, the lack of upregulation of any mPR isoform in the

absence of other steroid receptors suggests that the pattern of mPR expression in relation to Her-2/neu may mirror patterns seen between nuclear steroid receptors and Her-2/neu.

Conclusions

This study is the first to examine mPR expression in the human breast and clearly demonstrates that the mPRs are expressed in human breast tissue and that they are upregulated in nearly 80% of the tumors examined. While mPR transcript levels may be low compared to other steroid receptors, their expression patterns suggest that they may play a role in breast cancer biology. Of particular interest are the mPRα and mPRγ isoforms in that they positively correlate with nPR and CK19 upregulation in breast tumors respectively, both of which are associated with a positive prognosis. The patterns of mPR isoform expression in relation to other mPRs, nuclear steroid receptor isoforms, and prognosis warrant further study.

Literature Cited

Aaltomaa, S., Lipponen, P., Eskelinen, M., Kosma, V. M., Marin, S., Alhava, E., and Syrjanen, K. (1991). Hormone receptors as prognostic factors in female breast cancer. Ann Med *23*, 634-648.

Allegra, J. C., Lippman, M. E., Thompson, E. B., Simon, R., Barlock, A., Green, L., Huff, K. K., Do, H. M., Aitken, S. C., and Warren, R. (1980). Estrogen receptor status: an important variable in predicting response to endocrine therapy in metastatic breast cancer. Eur J Cancer *16*, 323-331.

Anderson, W. F., Chu, K. C., Chatterjee, N., Brawley, O., and Brinton , L. A. (2001). Tumor variants by hormone receptor expression in white patients with node-negative breast cancer from the surveillance, epidemiology, and end results database. J Clin Oncol *19*, 18-27.

Ariga, N., Suzuki, T., Moriya, T., Kimura, M., Inoue, T., Ohuchi, N., and Sasano, H. (2001). Progesterone receptor A and B isoforms in the human breast and its disorders. Jpn J Cancer Res *92*, 302-308.

Boonyaratanakornkit, V., McGowan, E., Sherman, L., Mancini, M. A., Cheskis, B. J., and Edwards, D. P. (2007). The role of extranuclear signaling actions of progesterone receptor in mediating progesterone regulation of gene expression and the cell cycle. Mol Endocrinol *21*, 359-375.

Boonyaratanakornkit, V., Scott, M. P., Ribon, V., Sherman, L., Anderson, S. M., Maller, J. L., Miller, W. T., and Edwards, D. P. (2001). Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. Mol Cell *8*, 269-280.

Carnevale, R. P., Proietti, C. J., Salatino, M., Urtreger, A., Peluffo, G., Edwards, D. P., Boonyaratanakornkit, V., Charreau, E. H., Bal de Kier Joffe, E., Schillaci, R., and Elizalde, P. V. (2007). Progestin effects on breast cancer cell proliferation, proteases activation, and in vivo development of metastatic phenotype all depend on progesterone receptor capacity to activate cytoplasmic signaling pathways. Mol Endocrinol *21*, 1335-1358.

Chlebowski, R. T., Hendrix, S. L., Langer, R. D., Stefanick, M. L., Gass, M., Lane, D., Rodabough, R. J., Gilligan, M. A., Cyr, M. G., Thomson, C. A.*, et al.* (2003). Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative Randomized Trial. Jama *289*, 3243-3253.

Crowe, J. P., Gordon, N. H., Hubay, C. A., Shenk, R. R., Zollinger, R. M., Brumberg, D. J., McGuire, W. L., and Shuck, J. M. (1991). Estrogen receptor determination and long term survival of patients with carcinoma of the breast. Surg Gynecol Obstet *173*, 273-278.

De Bessa, S. A., Salaorni, S., Patrao, D. F., Neto, M. M., Brentani, M. M., and Nagai, M. A. (2006). JDP1 (DNAJC12/Hsp40) expression in breast cancer and its association with estrogen receptor status. Int J Mol Med *17*, 363-367.

De Sombre, E. R., Smith, S., Block, G. E., Ferguson, D. J., and Jensen, E. V. (1974). Prediction of breast cancer response to endocrine therapy. Cancer Chemother Rep *58*.

Dunnwald, L., Rossing, M. A., and Li, C. I. (2007). Hormone receptor status, tumor characteristics, and prognosis: a prospective cohort of breast cancer patients. Breast Cancer Res *9*, R6.

Faivre, E., Skildum, A., Pierson-Mullany, L., and Lange, C. A. (2005). Integration of progesterone receptor mediated rapid signaling and nuclear actions in breast cancer cell models: role of mitogen-activated protein kinases and cell cycle regulators. Steroids *70*, 418-426.

Filardo, E., Quinn, J., Pang, Y., Graeber, C., Shaw, S., Dong, J., and Thomas, P. (2007). Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane. Endocrinology *148*, 3236-3245.

Filardo, E. J., and Thomas, P. (2005). GPR30: a seven-transmembrane-spanning estrogen receptor that triggers EGF release. Trends Endocrinol Metab *16*, 362-367.

Fink, L., Seeger, W., Ermert, L., Hanze, J., Stahl, U., Grimminger, F., Kummer, W., and Bohle, R. M. (1998). Real-time quantitative RT-PCR after laser-assisted cell picking. Nat Med *4*, 1329-1333.

Fisher, B., Redmond, C., Fisher, E. R., and Caplan, R. (1988). Relative worth of estrogen or progesterone receptor and pathologic characteristics of differentiation as indicators of prognosis in node negative breast cancer patients: fingings from National Surgical Adjuvant Breast and Bowel Project Protocol. J Clin Oncol *6*, 1076-1087.

Fuqua, S. A., Cui, Y., Lee, A. V., Osborne, C. K., and Horwitz, K. B. (2005). Insights into the role of progesterone receptors in breast cancer. J Clin Oncol *23*, 931-932; author reply 932-933.

Furmaniski, P., Saunders, D. E., Brooks, S. C., and M.A., R. (1980). the prognostic value of estrogen receptor determination in patients with primary breast cancer. Cancer *46*, 2794-2796.

Geyer, H., Eberl, M., and Hornberger, B. (1985). Influence of tissue heterogeneity on the determination of steroid receptors in breast cancer. J Cancer Res Clin Oncol *110*, 141-144.

Hopp, T. A., Weiss, H. L., Hilsenbeck, S. G., Cui, Y., Allred, D. C., Horwitz, K. B., and Fuqua, S. A. (2004a). Breast cancer patients with progesterone receptor PR-A-rich tumors have poorer disease-free survival rates. Clin Cancer Res *10*, 2751-2760.

Hopp, T. A., Weiss, H. L., Parra, I. S., Cui, Y., Osborne, C. K., and Fuqua, S. A. (2004b). Low levels of estrogen receptor beta protein predict resistance to tamoxifen therapy in breast cancer. Clin Cancer Res *10*, 7490-7499.

Horwitz, K. B., and McGuire, W. L. (1975). Predicting response to endocrine therapy in human breast cancer: A hypothesis. Science *189*.

Horwitz, K. B., Zava, D. T., Thilagar, A. K., Jensen, E. M., and McGuire, W. L. (1978). Steroid receptor analyses of nine human breast cancer cell lines. Cancer Res *38*, 2434-2437.

Howat, J., Harris, M., Swindell, R., and Barnes, D. (1985). The effect of oestrogen and progesterone receptors on recurrence and survival in patients with carcinoma of the breast. Br J Cancer *51*, 263-268.

Iakovlev, V. V., Goswami, R. S., Vecchiarelli, J., Arneson, N. C., and Done, S. J. (2008). Quantitative detection of circulating epithelial cells by Q-RT-PCR. Breast Cancer Res Treat *107*, 145-154.

Jensen, M. L., Kiaer, H., and Melsen, F. (1996). Medullary breast carcinoma vs. poorly differentiated ductal carcinoma: an immunohistochemical study with keratin 19 and oestrogen receptor staining. Histopathology *29*, 241-245.

Ji, X. Q., Sato, H., Tanaka, H., Konishi, Y., Fujimoto, T., Takahashi, O., and Tanaka, T. (2006). Real-time quantitative RT-PCR detection of disseminated endometrial tumor cells in peripheral blood and lymph nodes using the LightCycler System. Gynecol Oncol *100*, 355-360.

Karteris, E., Sevasti, Z., Pang, Y., Dong, J., Hillhouse, E. W., Randeva, H. S., and Thomas, P. (2006). Progesterone signaling in human myometrium through two novel membrane g protein-coupled receptors: potential role in functional progesterone withdrawl at term. Mol Endo *20*, 1519-1534.

Keshgegian, A. A. (1994). Biochemically estrogen receptor-negative, progesterone receptor-positive breast carcinoma. Immunocytochemical hormone receptors and prognostic factors. Arch Pathol Lab Med *118*, 240-244.

Keshgegian, A. A., and Cnaan, A. (1996). Estrogen receptor-negative, progesterone receptor-positive breast carcinoma: poor clinical outcome. Arch Pathol Lab Med *120*, 970-973.

Knight, W. A., 3rd, Osborne, C. K., Yochmowitz, M. G., and McGuire, W. L. (1980). Steroid hormone receptors in the management of human breast cancer. Ann Clin Res *12*, 202-207.

Konecny, G., Pauletti, G., Pegram, M., Untch, M., Dandekar, S., Aguilar, Z., Wilson, C., Rong, H. M., Bauerfeind, I., Felber, M.*, et al.* (2003). Quantitative association between HER-2/neu and steroid hormone receptors in hormone receptor-positive primary breast cancer. J Natl Cancer Inst *95*, 142-153.

Lange, C. A. (2007). Challenges to defining a role for progesterone in breast cancer. Steroids.

Lange, C. A., Richer, J. K., and Horwitz, K. B. (1999). Hypothesis: Progesterone primes breast cancer cells for cross-talk with proliferative or antiproliferative signals. Mol Endocrinol *13*, 829-836.

Lange, C. A., Richer, J. K., Shen, T., and Horwitz, K. B. (1998). Convergence of progesterone and epidermal growth factor signaling in breast cancer. Potentiation of mitogen-activated protein kinase pathways. J Biol Chem *273*, 31308-31316.

Larsimont, D., Lespagnard, L., Degeyter, M., and Heimann, R. (1994). Medullary carcinoma of the breast: a tumor lacking keratin 19. Histopathology *24*, 549-552.

Latil, A., Bieche, I., Vidaud, D., Lidereau, R., Berthon, P., Cussenot, O., and Vidaud, M. (2001). Evaluation of androgen, estrogen (ER alpha and ER beta), and progesterone receptor expression in human prostate cancer by real-time quantitative reverse transcription-polymerase chain reaction assays. Cancer Res *61*, 1919-1926.

Li, C. I., Weiss, N. S., Stanford, J. L., and Daling, J. R. (2000). Hormone replacement therapy in relation to risk of lobular and ductal breast carcinoma in middle-aged women. Cancer *88*, 2570-2577.

Lin, C. Y., Strom, A., Li Kong, S., Kietz, S., Thomsen, J. S., Tee, J. B., Vega, V. B., Miller, L. D., Smeds, J., Bergh, J., *et al.* (2007). Inhibitory effects of estrogen receptor beta on specific hormone-responsive gene expression and association with disease outcome in primary breast cancer. Breast Cancer Res *9*, R25.

McGowan, E. M., Russell, A. J., Boonyaratanakornkit, V., Saunders, D. N., Lehrbach, G. M., Sergio, C. M., Musgrove, E. A., Edwards, D. P., and Sutherland, R. L. (2007). Progestins reinitiate cell cycle progression in antiestrogen-arrested breast cancer cells through the B-isoform of progesterone receptor. Cancer Res *67*, 8942-8951.

Migliaccio, A., Piccolo, D., Castoria, G., Di Domenico, M., Bilancio, A., Lombardi, M., Gong, W., Beato, M., and Auricchio, F. (1998). Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. Embo J *17*, 2008-2018.

Moore, M. R. (2004). A rationale for inhibiting progesterone-related pathways to combat breast cancer. Curr Cancer Drug Targets *4*, 183-189.

Moore, M. R., Conover, J. L., and Franks, K. M. (2000). Progestin effects on long-term growth, death, and Bcl-xL in breast cancer cells. Biochem Biophys Res Commun *277*, 650-654.

Moore, M. R., Spence, J. B., Kiningham, K. K., and Dillon, J. L. (2006). Progestin inhibition of cell death in human breast cancer cell lines. J Steroid Biochem Mol Biol *98*, 218-227.

Nardulli, A. M., Greene, G. L., O'Malley, B. W., and Katzenellenbogen, B. S. (1988). Regulation of progesterone receptor messenger ribonucleic acid and protein levels

in MCF-7 cells by estradiol: Analysis of estrogen's effect on progesterone receptor synthesis and degradation. Endo *122*, 935-944.

Nicholson, S., Wright, C., Sainsbury, J. R., Halcrow, P., Kelly, P., Angus, B., Farndon, J. R., and Harris, A. L. (1990). Epidermal growth factor receptor (EGFr) as a marker for poor prognosis in node-negative breast cancer patients: neu and tamoxifen failure. J Steroid Biochem Mol Biol *37*, 811-814.

Olivotto, I. A., Truong, P. T., Speers, C. H., Bernstein, V., Allan, S. J., Kelly, S. J., and Lesperance, M. L. (2004). Time to stop progesterone receptor testing in breast cancer management. J Clin Oncol *22*, 1769-1770.

Ory, K., Lebeau, J., Levalois, C., Bishay, K., Fouchet, P., Allemand, I., Therwath, A., and Chevillard, S. (2001). Apoptosis inhibition mediated by medroxyprogesterone acetate treatment of breast cancer cell lines. Breast Cancer Res Treat *68*, 187-198.

Osborne, C. K., Yochmowitz, M. G., Knight, W. A., 3rd, and McGuire, W. L. (1980). The value of estrogen and progesterone receptors in the treatment of breast cancer. Cancer *46*, 2884-2888.

Parikh, R. R., Yang, Q., Higgins, S. A., and Haffty, B. G. (2008). Outcomes in young women with breast cancer of triple-negative phenotype: the prognostic significance of CK19 expression. Int J Radiat Oncol Biol Phys *70*, 35-42.

Parl, F. F., and Dupont, W. D. (1982). A retrospective cohort study of histologic risk factors in breast cancer patients. Cancer *50*, 2410-2416.

Parl, F. F., Schmidt, B. P., Dupont, W. D., and Wagner, R. K. (1984). Prognositc significance of estrogen receptor status in breast cancer in relation to tumor stage, azillary node metastasis, and histopathologic grading. Cancer *54*, 2237-2242.

Peiro, G., Adrover, E., Aranda, F. I., Peiro, F. M., Niveiro, M., and Sanchez-Paya, J. (2007). Prognostic implications of HER-2 status in steroid receptor-positive, lymph node-negative breast carcinoma. Am J Clin Pathol *127*, 780-786.

Perou, C. M., Sorlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A.*, et al.* (2000). Molecular portraits of human breast tumors. Nature *406*, 747-752.

Persson, I., Weiderpass, E., Bergkvist, L., Bergstrom, R., and Schairer, C. (1999). Risks of breast and endometrial cancer after estrogen and estrogen-progestin replacement. Cancer Causes Control *10*, 253-260.

Reiner, A., Reiner, G., Spona, J., Schemper, M., and Holzner, J. H. (1988). Histopathologic characterization of human breast cancer in correlation with estrogen receptor status. A comparison of immunocytochemical and biochemical analysis. Cancer *61*, 1149-1154.

Richer, J. K., Lange, C. A., Manning, N. G., Owen, G., Powell, R., and Horwitz, K. B. (1998). Convergence of progesterone with growth factor and cytokine signaling in breast cancer. Progesterone receptors regulate signal transducers and activators of transcription expression and activity. J Biol Chem *273*, 31317-31326.

Ross, R. K., Paganini-Hill, A., Wan, P. C., and Pike, M. C. (2000). Effect of hormone replacement therapy on breast cancer risk: estrogen versus estrogen plus progestin. J Natl Cancer Inst *92*, 328-332.

Schairer, C., Lubin, J., Troisi, R., Sturgeon, S., Brinton, L., and Hoover, R. (2000). Estrogen-progestin replacement and risk of breast cancer. Jama *284*, 691-694.

Shaw, J. A., Udokang, K., Mosquera, J. M., Chauhan, H., Jones, J. L., and Walker, R. A. (2002). Oestrogen receptors alpha and beta differ in normal human breast and breast carcinomas. J Pathol *198*, 450-457.

Shupnik, M. A. (2007). Estrogen receptor-beta: why may it influence clinical outcome in estrogen receptor-alpha positive breast cancer? Breast Cancer Res *9*, 107.

Skildum, A., Faivre, E., and Lange, C. A. (2005). Progesterone receptors induce cell cycle progression via activation of mitogen-activated protein kinases. Mol Endocrinol *19*, 327-339.

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science *235*, 177-182.

Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and et al. (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science *244*, 707-712.

Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S.*, et al.* (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A *98*, 10869-10874.

Su, L., Morgan, P. R., and Lane, E. B. (1996). Expression of cytokeratin messenger RNA versus protein in the normal mammary gland and in breast cancer. Hum Pathol *27*, 800-806.

Thomas, P., Pang, Y., Dong, J., Groenen, P., Kelder, J., de Vlieg, J., Zhu, Y., and Tubbs, C. (2007). Steroid and G protein binding characteristics of the seatrout and human

progestin membrane receptor alpha subtypes and their evolutionary origins. Endocrinology *148*, 705-718.

Thomas, P., Pang, Y., Filardo, E. J., and Dong, J. (2005). Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology *146*, 624-632.

Vares, G., Ory, K., Lectard, B., Levalois, C., Altmeyer-Morel, S., Chevillard, S., and Lebeau, J. (2004). Progesterone prevents radiation-induced apoptosis in breast cancer cells. Oncogene *23*, 4603-4613.

Varghese, C. (2007). The significance of oestrogen and progesterone receptors in breast cancer. J Clin Diag Res *3*, 198-203.

Visser, M., Jiwa, M., Horstman, A., Brink, A. A., Pol, R. P., Diest, P. V., Snijders, P. J., and Meijer, C. J. (2008). Intra-operative rapid diagnostic method based on CK19 mRNA expression for the detection of lymph node metastases in breast cancer. Int J Cancer.

von Maillot, K., Horke, W., and Prestele, H. (1982). Prognostic significance of the steroid receptor content in primary breast cancer. Arch Gynecol *231*, 185-190.

Wright, C., Nicholson, S., Angus, B., Sainsbury, J. R., Farndon, J., Cairns, J., Harris, A. L., and Horne, C. H. (1992). Relationship between c-erbB-2 protein product expression and response to endocrine therapy in advanced breast cancer. Br J Cancer *65*, 118-121.

Xu, R., Feiner, H., Li, P., Yee, H., Inghirami, G., Delgado, Y., and Perle, M. A. (2003). Differential amplification and overexpression of HER-2/neu, p53, MIB1, and estrogen receptor/progesterone receptor among medullary carcinoma, atypical

medullary carcinoma, and high-grade invasive ductal carcinoma of breast. Arch Pathol Lab Med *127*, 1458-1464.

Zhu, Y., Bond, J., and Thomas, P. (2003a). Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. Proc Natl Acad Sci U S A *100*, 2237-2242.

Zhu, Y., Rice, C. D., Pang, Y., Pace, M., and Thomas, P. (2003b). Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc Natl Acad Sci U S A *100*, 2231-2236.

Table 4-1: Primer sequences and fragment sizes used in quantitative RT-PCR

Primer Name	Sequence: Sense Anti-sense	Fragment Size (base pairs)
mPRα	5'cgctcttctggaagccgtacatctatg 5'cagcaggtgggtccagacattcac	100
mPRβ	5'agcctcctacatagatgctgccc 5'ggtgcctggttcacatgttcttca	194
mPRγ	5'cagctgtttcacgtgtgtgtgatcctg 5'gcacagaagtatggctccagctatctgag	120
PGRMC1	5'tctggactgcactgttgtccttg 5'gcaaacacctgttcctattctg	290
PR	5'cgcgctctaccctgcactc 5'tgaatccggcctcaggtagtt	121
CK19	5'gcactacagccactactacacga 5'ctcatgcgcagagcctgtt	159
B-actin	5'gcgggaaatcgtgcgtgacatt 5'gatggagttgaaggtagtttcgtg	305

Table 4-2: Description of biopsy samples.

Biopsy Designation	Age	Race	Tumor Type	Bloom/Richardson Grade
1	62	W	Infiltrative Ductal Carcinoma	II
2	30	UNK	Infiltrative Ductal Carcinoma	II
3	79	W	Infiltrative Ductal Carcinoma	III
4	64	B	Medullary Carcinoma	III
5	37	W	Infiltrative Ductal Carcinoma	II
6	80	W	Infiltrative Ductal Carcinoma	III
7	27	W	Infiltrative Ductal Carcinoma	III
8	50	UNK	Infiltrative Ductal Carcinoma	
9	60	UNK	Infiltrative Ductal Carcinoma	III
10	51	UNK	Infiltrative Ductal Carcinoma	I
11	47	UNK	Pleomorphic Lobular Carcinoma	III
12	75	UNK	Infiltrative Ductal Carcinoma	
13	37	UNK	Infiltrative Ductal Carcinoma	II

Table 4-3: Gene regulation between paired normal and malignant samples. (+) denotes a ratio of malignant to normal gene expression greater than 1.2. (–) denotes a ratio of malignant to normal gene expression less than 1.2.

Biopsy Designation	CK19	mPRα	mPRβ	mPRγ	ERα	ERβ	nPR	PGRMC1
1	-	+	+	+	+	-	+	-
2	+	+	-	+	-	-	+	+
3	+	+	-	+	+	-	+	-
4	+	-	-	-	-	-	-	-
5	+	+	+	+	+	-	+	-
6	+	+	-	+	+	-	+	-
7	+	-	-	-	-	-	-	-
8	+	+	+	+	+	+	+	+
9	-	-	+	-	+	-	-	+
10	+	-	-	+	-	+	-	+
11	+	-	-	+	-	-	-	+
12	+	-	+	+	-	-	+	-
13	-	-	-	-	-	-	-	-

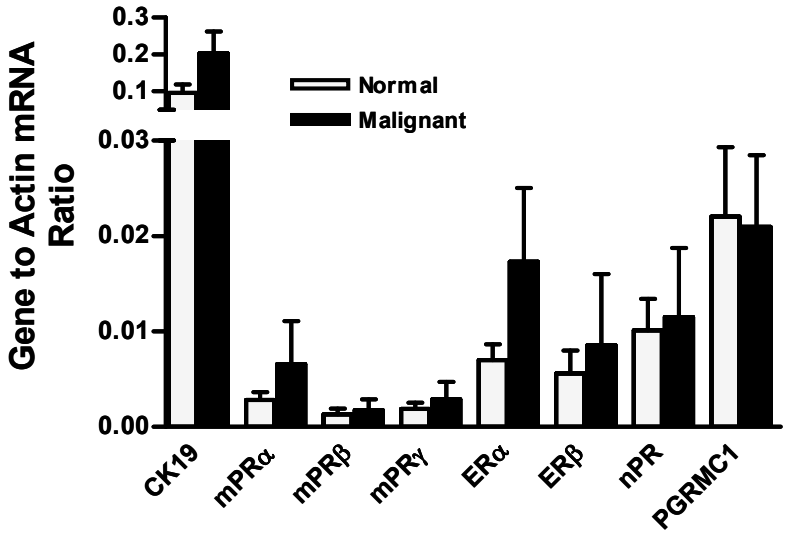

Figure 4-1: Average gene to actin ratio in normal and malignant tissues. Data represent average ± SEM gene to actin ratio for all measured genes grouped according to disease state.

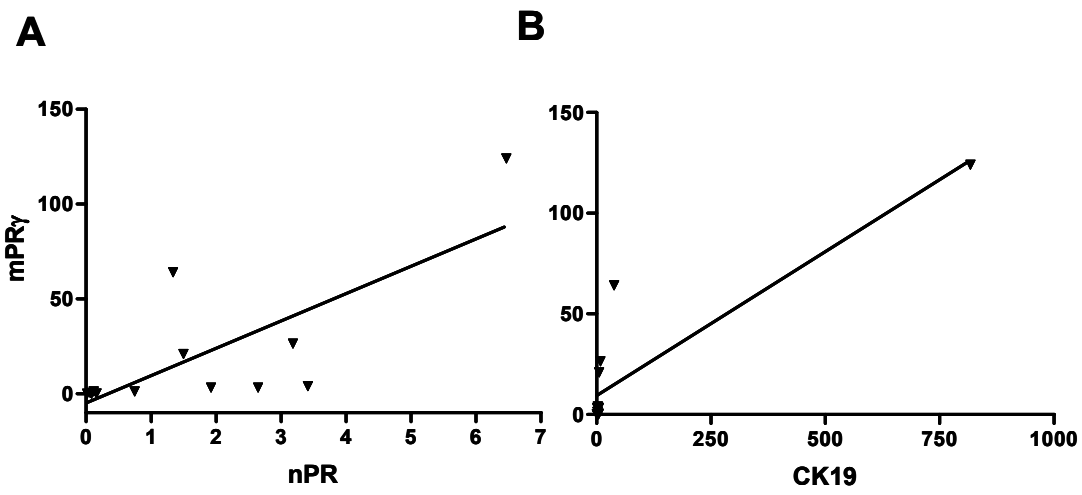

Figure 4-2: mPRγ linear correlation with nPR and CK19. Data represent the ratio of malignant to normal gene expression for each biopsy pair. A. Linear regression analysis indicates a positive slope of m = 14.42 ± 3.846. The deviation from m=0 was significant with P<0.005 and r2= 0.5609. B. Linear regression analysis indicates a positive slope of m = 0.1430 ± 0.02262. The deviation from m=0 was significant with P<0.0001 and r^2= 0.784

Chapter 5

Summary and Conclusions

The results from the present study support the hypotheses that the mechanism of mPR signaling and the biological function of the mPR in the somatic cells of female reproductive tissues are conserved across vertebrates and suggest that the mPRs may have significance in human disease. In addition, the current study proposes a novel function for the mPR in female reproductive tissues. Studies on the signaling of mPR in both the teleost ovarian follicle and immortalized human breast cancer cell lines demonstrate that progestin acts through the mPRs to activate inhibitory G proteins, decrease intracellular cAMP and activate ERK. mPR activation of inhibitory G protein signaling pathways in both fish and humans indicates that the mechanism of mPR intracellular signaling appears to be conserved in female tissues across vertebrate classes. In addition, the mechanism of mPR signaling appears to be conserved in female reproductive tissues as mPR signaling through inhibitory G proteins has now been shown in the ovarian follicle and breast by the current study, and in the oocyte and myometrium by previous studies.

The present study also suggests a novel function for the mPR in the somatic cells of female reproductive tissues. Studies in both fish ovarian follicle cells and immortalized breast cancer cells demonstrate progestin inhibition of apoptosis in a manner consistent with mPR mediation. Although progestin inhibition of apoptosis mediates two physiologically different results, mediation of spawning synchronization in the fish ovarian follicle and promoting the diseased, neoplastic nature of immortalized

breast cancer cells, the actions of progestin via mPR activation appears to be the same. While the complete intracellular signaling cascade leading from the mPR to inhibition of DNA fragmentation has not been elucidated and requires further study, it is plausible that activation of ERK and potentially Akt by the mPR are involved.

The findings from this study also strongly suggest a significant role for the mPR in breast cancer biology. In addition to mPR involvement in progesterone's anti-apoptotic effect in immortalized cells enabling diseased cells to continue to survive and grow in the absence of growth factor signals, the mPRs are upregulated in the malignant tissue from the majority of breast cancer patients examined. Of the three predominant human mPR isoforms (mPRα, mPRβ and mPRγ) mPRγ, about which little is known, appears to be the most prevalent in breast tumors and may suggest a role for this isoform in cancer biology. mPRγ upregulation correlated with nPR expression and CK19 suggesting a role for the mPRs in steroid responsive tumors of epithelial origin.

This series of studies show that mPR signaling and function are conserved in female tissues across vertebrate classes and suggest a novel function for the mPR. While further investigations into the mechanism of mPR mediation of progestin's protective affect and the role of the mPRs in breast tumors are needed, the current study is a demonstration that studies in fish can lead us to discoveries in human health.

Bibliography

Aaltomaa, S., Lipponen, P., Eskelinen, M., Kosma, V. M., Marin, S., Alhava, E., and Syrjanen, K. (1991). Hormone receptors as prognostic factors in female breast cancer. Ann Med *23*, 634-648.

Abraham, M., and Shaham, S. (2004). Death without caspases, caspases without death. TRENDS Cell Biol *14*, 184-193.

Alkhalaf, M., El-Mowafy, A., and Karam, S. (2002). Growth inhibition of MCF-7 human breast cancer cells by progesterone is associated with cell differentiation and phosphorylation of Akt protein. Eur J Cancer Prev *11*, 481-488.

Allegra, J. C., Lippman, M. E., Thompson, E. B., Simon, R., Barlock, A., Green, L., Huff, K. K., Do, H. M., Aitken, S. C., and Warren, R. (1980). Estrogen receptor status: an important variable in predicting response to endocrine therapy in metastatic breast cancer. Eur J Cancer *16*, 323-331.

Anderson, W. F., Chu, K. C., Chatterjee, N., Brawley, O., and Brinton , L. A. (2001). Tumor variants by hormone receptor expression in white patients with node-negative breast cancer from the surveillance, epidemiology, and end results database. J Clin Oncol *19*, 18-27.

Ariga, N., Suzuki, T., Moriya, T., Kimura, M., Inoue, T., Ohuchi, N., and Sasano, H. (2001). Progesterone receptor A and B isoforms in the human breast and its disorders. Jpn J Cancer Res *92*, 302-308.

Ashley, R. L., Clay, C. M., Farmerie, T. A., Niswender, G. D., and Nett, T. M. (2006). Cloning and characterization of an ovine intracellular seven transmembrane

receptor for progesterone that mediates calcium mobilization. Endo *147*, 4151-4159.

Baldi, E., Luconi, M., Bonaccorsi, L., and Forti, G. (1998). Nongenomic effects of progesterone on spermatozoa: mechanisms of signal transduction and clinical implications. Front Biosci *3*, D1051-1059.

Ballare, C., Uhrig, M., Bechtold, T., Sancho, E., Di Domenico, M., Migliaccio, A., Auricchio, F., and Beato, M. (2003). Two domains of the progesterone receptor interact with the estrogen receptor and are required for progesterone activation of the c-Src/Erk pathway in mammalian cells. Mol Cell Biol *23*, 1994-2008.

Bardon, S., Vignon, F., Montcourrier, P., and Rochefort, H. (1987). Steroid receptor-mediated cytotoxicity of an antiestrogen and an antiprogestin in breast cancer cells. Cancer Res *47*, 1441-1448.

Basu, A., and Rowan, B. G. (2005). Genes related to estrogen action in reproduction and breast cancer. Front Biosci *10*, 2346-2372.

Benninghoff, A. D., and Thomas, P. (2006). Gonadotropin regulation of testosterone production by primary cultured theca and granulosa cells of Atlantic croaker: I. Novel role of CaMKs and interactions between calcium- and adenylyl cyclase-dependent pathways. Gen Comp Endocrinol *147*, 276-287.

Blagosklonny, M. (2000). Cell death beyond apoptosis. Leukemia *14*, 1502-1508.

Boonyaratanakornkit, V., McGowan, E., Sherman, L., Mancini, M. A., Cheskis, B. J., and Edwards, D. P. (2007). The role of extranuclear signaling actions of progesterone receptor in mediating progesterone regulation of gene expression and the cell cycle. Mol Endocrinol *21*, 359-375.

Boonyaratanakornkit, V., Scott, M. P., Ribon, V., Sherman, L., Andersson, M., Maller, J. L., Miller, W. T., and Edwards, D. P. (2001). Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. Mol Cell *8*, 269-280.

Borner, C., and Monney, L. (1999). Apoptosis without caspases: an inefficient molecular guillotine? Cell Death Diff *6*, 497-507.

Cahill, M. (2007). Progesterone receptor membrane component 1: An integrative review. Steroid Biochem Mol Bio *105*, 16-36.

Cai, Z., and Stocco, C. O. (2005). Expression and regulation of progestin membrane receptors in the rat corpus luteum. Endocrinology *146*, 5522-5532.

Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998). Regulation of cell death protease caspase-9 by phosphorylation. Science *282*, 1318-1321.

Carnevale, R. P., Proietti, C. J., Salatino, M., Urtreger, A., Peluffo, G., Edwards, D. P., Boonyaratanakornkit, V., Charreau, E. H., Bal de Kier Joffe, E., Schillaci, R., and Elizalde, P. V. (2007). Progestin effects on breast cancer cell proliferation, proteases activation, and in vivo development of metastatic phenotype all depend on progesterone receptor capacity to activate cytoplasmic signaling pathways. Mol Endocrinol *21*, 1335-1358.

Chaffin, C. L., and Stouffer, R. L. (2000). Role of gonadotrophins and progesterone in the regulation of morphological remodelling and atresia in the monkey peri-ovulatory follicle. Hum Reprod *15*, 2489-2495.

Chlebowski, R. T., Hendrix, S. L., Langer, R. D., Stefanick, M. L., Gass, M., Lane, D., Rodabough, R. J., Gilligan, M. A., Cyr, M. G., Thomson, C. A.*, et al.* (2003). Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative Randomized Trial. Jama *289*, 3243-3253.

Crowe, J. P., Gordon, N. H., Hubay, C. A., Shenk, R. R., Zollinger, R. M., Brumberg, D. J., McGuire, W. L., and Shuck, J. M. (1991). Estrogen receptor determination and long term survival of patients with carcinoma of the breast. Surg Gynecol Obstet *173*, 273-278.

Daniel, A., Qiu, M., Faivre, E., Ostrander, J., Skildum, A., and Lange, C. (2007). Linkage of progestin and epidermal growth factor signaling: phosphorylation of progesterone receptors mediates transcriptional hypersensitivity and increased ligand-independent breast cancer cell growth. Steroids *72*, 188-201.

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*, 231-241.

De Bessa, S. A., Salaorni, S., Patrao, D. F., Neto, M. M., Brentani, M. M., and Nagai, M. A. (2006). JDP1 (DNAJC12/Hsp40) expression in breast cancer and its association with estrogen receptor status. Int J Mol Med *17*, 363-367.

De Sombre, E. R., Smith, S., Block, G. E., Ferguson, D. J., and Jensen, E. V. (1974). Prediction of breast cancer response to endocrine therapy. Cancer Chemother Rep *58*.

Dressing, G. E., and Thomas, P. (2007). Identification of membrane progestin receptors in human breast cancer cell lines and biopsies and their potential involvement in breast cancer. Steroids *72*, 111-115.

Drummond, A. E. (2006). The role of steroids in follicular growth. Reprod Biol Endocrinol *4*, 16.

Drummond, C. D., Bazzoli, N., Rizzo, E., and Sato, Y. (2000). Postovulatory follicle: a model for experimental studies of programmed cell death or apoptosis in teleosts. J Exp Zool *287*, 176-182.

Duffy, D. M., Wells, T. R., Haluska, G. J., and Stouffer, R. L. (1997). The ratio of progesterone receptor isoforms changes in the monkey corpus luteum during the luteal phase of the menstrual cycle. Biol Reprod *57*, 693-699.

Dunnwald, L., Rossing, M. A., and Li, C. I. (2007). Hormone receptor status, tumor characteristics, and prognosis: a prospective cohort of breast cancer patients. Breast Cancer Res *9*, R6.

Engmann, L., Losel, R., Wehling, M., and Peluso, J. J. (2006). Progesterone regulation of human granulosa/luteal cell viability by an RU486-independent mechanism. J Clin Endocrinol Metab *91*, 4962-4968.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. Science *240*, 889-895.

Faivre, E., Skildum, A., Pierson-Mullany, L., and Lange, C. A. (2005). Integration of progesterone receptor mediated rapid signaling and nuclear actions in breast cancer cell models: role of mitogen-activated protein kinases and cell cycle regulators. Steroids *70*, 418-426.

Falkenstein, E., Eisen, C., Schmieding, K., Krautkramer, M., Stein, C., Losel, R., and Wehling, M. (2001). Chemical modification and structural analysis of the progesterone membrane binding protein from porcine liver membranes. Mol Cell Biochem *218*, 71-79.

Falkenstein, E., Heck, M., Gerdes, D., Grube, D., Christ, M., Weigel, M., Buddhikot, M., Meizel, S., and Wehling, M. (1999). Specific progesterone binding to a membrane protein and related nongenomic effects on Ca2+-fluxes in sperm. Endocrinology *140*, 5999-6002.

Falkenstein, E., Schmieding, K., Lange, A., Meyer, C., Gerdes, D., Welsch, U., and Wehling, M. (1998). Localization of a putative progesterone membrane binding protein in porcine hepatocytes. Cell Mol Biol (Noisy-le-grand) *44*, 571-578.

Feng, Z., Marti, A., Jehn, B., Altermatt, H. J., Chicaiza, G., and Jaggi, R. (1995). Glucocorticoid and progesterone inhibit involution and programmed cell death in the mouse mammary gland. J Cell Biol *131*, 1095-1103.

Filardo, E., Quinn, J., Pang, Y., Graeber, C., Shaw, S., Dong, J., and Thomas, P. (2007). Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane. Endocrinology *148*, 3236-3245.

Filardo, E. J., and Thomas, P. (2005). GPR30: a seven-transmembrane-spanning estrogen receptor that triggers EGF release. Trends Endocrinol Metab *16*, 362-367.

Fink, L., Seeger, W., Ermert, L., Hanze, J., Stahl, U., Grimminger, F., Kummer, W., and Bohle, R. M. (1998). Real-time quantitative RT-PCR after laser-assisted cell picking. Nat Med *4*, 1329-1333.

Fisher, B., Redmond, C., Fisher, E. R., and Caplan, R. (1988). Relative worth of estrogen or progesterone receptor and pathologic characteristics of differentiation as indicators of prognosis in node negative breast cancer patients: fingings from National Surgical Adjuvant Breast and Bowel Project Protocol. J Clin Oncol *6*, 1076-1087.

Formby, B., and Wiley, T. S. (1998). Progesterone inhibits growth and induces apoptosis in breast cancer cells: inverse effects on Bcl-2 and p53. Ann Clin Lab Sci *28*, 360-369.

Formby, B., and Wiley, T. S. (1999). Bcl-2, survivin and variant CD44 v7-v10 are downregulated and p53 is upregulated in breast cancer cells by progesterone: inhibition of cell growth and induction of apoptosis. Mol Cell Biochem *202*, 53-61.

Fortune, J. E., and Vincent, S. E. (1983). Progesterone inhibits the induction of aromatase activity in rat granulosa cells in vitro. Biol Reprod *28*, 1078-1089.

Freshney, R. I. (1994). Culture of Animal Cells: A manual of basic technique. In (New York, Wiley-Liss).

Fuqua, S. A., Cui, Y., Lee, A. V., Osborne, C. K., and Horwitz, K. B. (2005). Insights into the role of progesterone receptors in breast cancer. J Clin Oncol *23*, 931-932; author reply 932-933.

Furmaniski, P., Saunders, D. E., Brooks, S. C., and M.A., R. (1980). the prognostic value of estrogen receptor determination in patients with primary breast cancer. Cancer *46*, 2794-2796.

Gerdes, D., Wehling, M., Leube, B., and Falkenstein, E. (1998). Cloning and tissue expression of two putative steroid membrane receptors. Biol Chem *379*, 907-911.

Geyer, H., Eberl, M., and Hornberger, B. (1985). Influence of tissue heterogeneity on the determination of steroid receptors in breast cancer. J Cancer Res Clin Oncol *110*, 141-144.

Hamabe, W., Fukushima, N., Yoshida, A., and Ueda, H. (2000). Serum-free induced neuronal apoptosis-like cell death is independent of caspase activity. Mol Brain Res *78*, 186-191.

Hanna, R., Pang, Y., Thomas, P., and Zhu, Y. (2006). Cell-surface expression, progestin binding, and rapid nongenomic signaling of zebrafish membrane progestin receptors alpha and beta in transfected cells. J Endocrinol *190*, 247-260.

Harris, C. (2007) Examination of the role of different classes of progesterone receptors in oocyte maturation in zebrafish, Danio rerio, Masters of Science, University of Texas, Port Aransas.

Henson, E. S., and Gobson, S. B. (2006). Surviving cell death through epidermal growth factor (EGF) signal transduction pathways: Implications for cancer therapy. Cellular Signaling *18*, 2089-2097.

Hopp, T. A., Weiss, H. L., Hilsenbeck, S. G., Cui, Y., Allred, D. C., Horwitz, K. B., and Fuqua, S. A. (2004a). Breast cancer patients with progesterone receptor PR-A-rich tumors have poorer disease-free survival rates. Clin Cancer Res *10*, 2751-2760.

Hopp, T. A., Weiss, H. L., Parra, I. S., Cui, Y., Osborne, C. K., and Fuqua, S. A. (2004b). Low levels of estrogen receptor beta protein predict resistance to tamoxifen therapy in breast cancer. Clin Cancer Res *10*, 7490-7499.

Horwitz, K. B., and McGuire, W. L. (1975). Predicting response to endocrine therapy in human breast cancer:A hypothesis. Science *189*.

Horwitz, K. B., Zava, D. T., Thilagar, A. K., Jensen, E. M., and McGuire, W. L. (1978). Steroid receptor analyses of nine human breast cancer cell lines. Cancer Res *38*, 2434-2437.

Howat, J., Harris, M., Swindell, R., and Barnes, D. (1985). The effect of oestrogen and progesterone receptors on recurrence and survival in patients with carcinoma of the breast. Br J Cancer *51*, 263-268.

Iakovlev, V. V., Goswami, R. S., Vecchiarelli, J., Arneson, N. C., and Done, S. J. (2008). Quantitative detection of circulating epithelial cells by Q-RT-PCR. Breast Cancer Res Treat *107*, 145-154.

Ikeda, K., and Inoue, S. (2004). Estrogen receptors and their downstream targets in cancer. Arch Histol Cytol *67*, 435-442.

Jensen, M. L., Kiaer, H., and Melsen, F. (1996). Medullary breast carcinoma vs. poorly differentiated ductal carcinoma: an immunohistochemical study with keratin 19 and oestrogen receptor staining. Histopathology *29*, 241-245.

Ji, X. Q., Sato, H., Tanaka, H., Konishi, Y., Fujimoto, T., Takahashi, O., and Tanaka, T. (2006). Real-time quantitative RT-PCR detection of disseminated endometrial tumor cells in peripheral blood and lymph nodes using the LightCycler System. Gynecol Oncol *100*, 355-360.

Johnson, A. L. (2003). Intracellular mechanisms regulating cell survival in ovarian follicles. Animal Repro Sci *78*, 185-201.

Josefsberg Ben-Yehoshua, L., Lewellyn, A., Thomas, P., and Maller, J. (2007). The role of *Xenopus* membrane progesterone receptor β in mediating the effect of progesterone on oocyte maturation. Mol Endocrinol *21*, 664-673.

Kandouz, M., Lombet, A., Perrot, J. Y., Jacob, D., Carvajal, S., Kazem, A., Rostene, W., Therwath, A., and Gompel, A. (1999). Proapoptotic effects of antiestrogens, progestins and androgen in breast cancer cells. J Steroid Biochem Mol Biol *69*, 463-471.

Kandouz, M., Siromachkova, M., Jacob, D., Chretien Marquet, B., Therwath, A., and Gompel, A. (1996). Antagonism between estradiol and progestin on Bcl-2 expression in breast-cancer cells. Int J Cancer *68*, 120-125.

Karteris, E., Zervou, S., Pang, Y., Dong, J., Hillhouse, E., Randeva, H., and Thomas, P. (2007). Progesterone signaling in human myometrium through two novel membrane G protein coupled receptors: Potential role in functional progesterone withdrawl at term. Mol Endocrinol *20*, 1519-1534.

Kaslow, H. R., Lim, L. K., Moss, J., and Lesikar, D. D. (1987). Structure-activity analysis of the activation of pertussis toxin. Biochemistry *26*, 123-127.

Kazeto, Y., Goto-Kazeto, R., Thomas, P., and Trant, J. M. (2005). Molecular characterization of three forms of putative membrane-bound progestin receptors and their tissue-distribution in channel catfish, Ictalurus punctatus. J Mol Endocrinol *34*, 781-791.

Keshgegian, A. A. (1994). Biochemically estrogen receptor-negative, progesterone receptor-positive breast carcinoma. Immunocytochemical hormone receptors and prognostic factors. Arch Pathol Lab Med *118*, 240-244.

Keshgegian, A. A., and Cnaan, A. (1996). Estrogen receptor-negative, progesterone receptor-positive breast carcinoma: poor clinical outcome. Arch Pathol Lab Med *120*, 970-973.

Knight, W. A., 3rd, Osborne, C. K., Yochmowitz, M. G., and McGuire, W. L. (1980). Steroid hormone receptors in the mangement of human breast cancer. Ann Clin Res *12*, 202-207.

Konecny, G., Pauletti, G., Pegram, M., Untch, M., Dandekar, S., Aguilar, Z., Wilson, C., Rong, H. M., Bauerfeind, I., Felber, M.*, et al.* (2003). Quantitative association between HER-2/neu and steroid hormone receptors in hormone receptor-positive primary breast cancer. J Natl Cancer Inst *95*, 142-153.

Lange, C., Shen, T., and Horwitz, K. B. (2000). Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. Proc Natl Acad Sci U S A *97*, 1032-1037.

Lange, C. A. (2007). Challenges to defining a role for progesterone in breast cancer. Steroids.

Lange, C. A., Richer, J. K., and Horwitz, K. B. (1999). Hypothesis: Progesterone primes breast cancer cells for cross-talk with proliferative or antiproliferative signals. Mol Endocrinol *13*, 829-836.

Lange, C. A., Richer, J. K., Shen, T., and Horwitz, K. B. (1998). Convergence of progesterone and epidermal growth factor signaling in breast cancer. Potentiation of mitogen-activated protein kinase pathways. J Biol Chem *273*, 31308-31316.

Larmonier, N., Billerey, C., Rebe, C., Parcellier, A., Moutet, M., Fromentin, A., Kroemer, G., Garrido, C., Solary, E., Martin, F., and Bonnotte, B. (2002). An atypoical caspase-independent death pathway for an immunogenic cancer cell line. Oncogene *21*, 6091-6100.

Larsimont, D., Lespagnard, L., Degeyter, M., and Heimann, R. (1994). Medullary carcinoma of the breast: a tumour lacking keratin 19. Histopathology *24*, 549-552.

Latil, A., Bieche, I., Vidaud, D., Lidereau, R., Berthon, P., Cussenot, O., and Vidaud, M. (2001). Evaluation of androgen, estrogen (ER alpha and ER beta), and progesterone receptor expression in human prostate cancer by real-time quantitative reverse transcription-polymerase chain reaction assays. Cancer Res *61*, 1919-1926.

Li, C. I., Weiss, N. S., Stanford, J. L., and Daling, J. R. (2000). Hormone replacement therapy in relation to risk of lobular and ductal breast carcinoma in middle-aged women. Cancer *88*, 2570-2577.

Lin, C. Y., Strom, A., Li Kong, S., Kietz, S., Thomsen, J. S., Tee, J. B., Vega, V. B., Miller, L. D., Smeds, J., Bergh, J.*, et al.* (2007). Inhibitory effects of estrogen receptor beta on specific hormone-responsive gene expression and association with disease outcome in primary breast cancer. Breast Cancer Res *9*, R25.

Lin, H., Chen, C., Li, X., and Chen, B. D. (2002). Activation of the MEK/MAPK pathway is involved in bryostatin1-induced monocytic differenciation and up-regulation of X-linked inhibitor of apoptosis protein. Exp Cell Res *272*, 192-198.

Lyons, T. J., Villa, N. Y., Regalla, L. M., Kupchak, B. R., Vagstad, A., and Eide, D. J. (2004). Metalloregulation of yeast membrane steroid receptor homologs. Proc Natl Acad Sci U S A *101*, 5506-5511.

Maller, J. L., and Krebs, E. G. (1980). Regulation of oocyte maturation. Curr Top Cell Regul *16*, 271-311.

Mathiasen, I., Lademann, U., and Jäättelä, M. (1999). Apoptosis induced by vitamin D compounds in breast cancer cells is inhibited by Bcl-2 but does not involve known caspases or p53. Cancer Res *59*, 4848-4856.

Mathiasen, I., Sergeev, I., Bastholm, L., Elling, F., Norman, A., and Jäättelä, M. (2002). Calcium and calpain as key mediators of apopotsis-like death induced by vitamin D compounds in breast cancer cells. J Biol Chem *277*, 30738-30745.

Maybin, J. A., and Duncan, W. C. (2004). The human corpus luteum: which cells have progesterone receptors? Reproduction *128*, 423-431.

McGowan, E. M., Russell, A. J., Boonyaratanakornkit, V., Saunders, D. N., Lehrbach, G. M., Sergio, C. M., Musgrove, E. A., Edwards, D. P., and Sutherland, R. L. (2007). Progestins reinitiate cell cycle progression in antiestrogen-arrested breast cancer cells through the B-isoform of progesterone receptor. Cancer Res *67*, 8942-8951.

Medina, D. (2005). Mammary developmental fate and breast cancer risk. Endocr-Relat Cancer *12*, 483-495.

Meyer, C., Schmid, R., Schmieding, K., Falkenstein, E., and Wehling, M. (1998). Characterization of high affinity progesterone-binding membrane proteins by anti-peptide antiserum. Steroids *63*, 111-116.

Meyer, C., Schmid, R., Scriba, P. C., and Wehling, M. (1996). Purification and partial sequencing of high-affinity progesterone-binding site(s) from porcine liver membranes. Eur J Biochem *239*, 726-731.

Migliaccio, A., Castoria, G., Di Domenico, M., de Falco, A., Bilancio, A., Lombardi, M., Barone, M. V., Ametrano, D., Zannini, M. S., Abbondanza, C., and Auricchio, F. (2000). Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation. Embo J *19*, 5406-5417.

Migliaccio, A., Piccolo, D., Castoria, G., Di Domenico, M., Bilancio, A., Lombardi, M., Gong, W., Beato, M., and Auricchio, F. (1998). Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. Embo J *17*, 2008-2018.

Misao, R., Nakanishi, Y., Fujimoto, J., and Tamaya, T. (1998a). Steroid receptor mRNA levels in human corpus luteum. Horm Res *50*, 155-159.

Misao, R., Nakanishi, Y., Iwagaki, S., Fujimoto, J., and Tamaya, T. (1998b). Expression of progesterone receptor isoforms in corpora lutea of human subjects: correlation with serum oestrogen and progesterone concentrations. Mol Hum Reprod *4*, 1045-1052.

Moore, M. R. (2004). A rationale for inhibiting progesterone-related pathways to combat breast cancer. Curr Cancer Drug Targets *4*, 183-189.

Moore, M. R., Conover, J. L., and Franks, K. M. (2000). Progestin effects on long-term growth, death, and Bcl-xL in breast cancer cells. Biochem Biophys Res Commun *277*, 650-654.

Moore, M. R., Spence, J. B., Kiningham, K. K., and Dillon, J. L. (2006). Progestin inhibition of cell death in human breast cancer cell lines. J Steroid Biochem Mol Biol *98*, 218-227.

Mourot, B., Nguyen, T., Fostier, A., and Bobe, J. (2006). Two unrelated putative membrane-bound progestin receptors, progesterone membrane receptor component 1 (PGMRC1) and membrane progestin receptor (mPR) beta, are expressed in the rainbow trout oocyte and exhibit similar ovarian expression patterns. Repro Bio Endo *4*.

Mussche, S., and D'Herde, K. (2001). Contribution of progesterone, follicle stimulating hormone and glucocorticoids in survival of serum-free cultured granulosa cell explants. J Endo *169*, 321-331.

Nagahama, Y., Yoshikuni, M., Yamashita, M., Tokumoto, T., and Katsu, Y. (1995). Regulation of oocyte growth and maturation in fish. Curr Top Cell Regul *30*, 103-145.

Nardulli, A. M., Greene, G. L., O'Malley, B. W., and Katzenellenbogen, B. S. (1988). Regulation of progesterone receptor messenger ribonucleic acid and protein levels in MCF-7 cells by estradiol: Analysis of estrogen's effect on progesterone receptor synthesis and degradation. Endo *122*, 935-944.

Natraj, U., and Richards, J. (1993). Hormonal regulation, localization, and functional activity of the progesterone receptor in granulosa cells of rat preovulatory follicles. Endocrinology *133*, 761-769.

Nicholson, S., Wright, C., Sainsbury, J. R., Halcrow, P., Kelly, P., Angus, B., Farndon, J. R., and Harris, A. L. (1990). Epidermal growth factor receptor (EGFr) as a marker for poor prognosis in node-negative breast cancer patients: neu and tamoxifen failure. J Steroid Biochem Mol Biol *37*, 811-814.

Nolte, I., Jeckel, D., Wieland, F. T., and Sohn, K. (2000). Localization and topology of ratp28, a member of a novel family of putative steroid-binding proteins. Biochim Biophys Acta *1543*, 123-130.

Olivotto, I. A., Truong, P. T., Speers, C. H., Bernstein, V., Allan, S. J., Kelly, S. J., and Lesperance, M. L. (2004). Time to stop progesterone receptor testing in breast cancer management. J Clin Oncol *22*, 1769-1770.

Ory, K., Lebeau, J., Levalois, C., Bishay, K., Fouchet, P., Allemand, I., Therwath, A., and Chevillard, S. (2001). Apoptosis inhibition mediated by medroxyprogesterone acetate treatment of breast cancer cell lines. Breast Cancer Res Treat *68*, 187-198.

Osborne, C. K., Yochmowitz, M. G., Knight, W. A., 3rd, and McGuire, W. L. (1980). The value of estrogen and progesterone receptors in the treatment of breast cancer. Cancer *46*, 2884-2888.

Pace, M., and Thomas, P. (2005a). Steroid-induced oocyte maturation in Atlantic croaker (*Micropogonias undulatus*) is dependent on activation of the phosphatidylinositol 3-kinase/Akt signal transduction pathway. Bio Reprod *73*, 988-996.

Pace, M. C., and Thomas, P. (2005b). Activation of a pertussis toxin-sensitive, inhibitory G-protein is necessary for steroid-mediated oocyte maturation in spotted seatrout. Dev Biol *285*, 70-79.

Parikh, R. R., Yang, Q., Higgins, S. A., and Haffty, B. G. (2008). Outcomes in young women with breast cancer of triple-negative phenotype: the prognostic significance of CK19 expression. Int J Radiat Oncol Biol Phys *70*, 35-42.

Parl, F. F., and Dupont, W. D. (1982). A retrospective cohort study of histologic risk factors in breast cancer patients. Cancer *50*, 2410-2416.

Parl, F. F., Schmidt, B. P., Dupont, W. D., and Wagner, R. K. (1984). Prognositc significance of estrogen receptor status in breast cancer in relation to tumor stage, azillary node metastasis, and histopathologic grading. Cancer *54*, 2237-2242.

Patiño, R., and Thomas, P. (1990). Characterization of membrane receptor activity for 17α,20β,21-tryhydroxy-4-pregnen-3-one in ovaries of spotted seatrout (*Cynoscion nebulosus*). Gen Comp Endocrinol *78*, 204-217.

Pedram, A., Razandi, M., Sainson, R., Kim, J., Hughes, C., and Levin, E. (2007). A conserved mechanism for steroid receptor translocation to the plasma membrane. J Biol Chem *282*, 22278-22288.

Peiro, G., Adrover, E., Aranda, F. I., Peiro, F. M., Niveiro, M., and Sanchez-Paya, J. (2007). Prognostic implications of HER-2 status in steroid receptor-positive, lymph node-negative breast carcinoma. Am J Clin Pathol *127*, 780-786.

Peluso, J., Fernandez, G., Pappalardo, A., and White, B. (2001). Characterization of a putative membrane receptor for progesterone in rat granulosa cells. Bio Rep *65*, 94-101.

Peluso, J., Pappalardo, A., Losel, R., and Wehling, M. (2006). Progesterone membrane receptor component 1 expression in the immature rat ovary and its role in mediating progesterone's antiapoptotic action. Endocrinology *147*, 3133-3140.

Perou, C. M., Sorlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A.*, et al.* (2000). Molecular portraits of human breast tumours. Nature *406*, 747-752.

Persson, I., Weiderpass, E., Bergkvist, L., Bergstrom, R., and Schairer, C. (1999). Risks of breast and endometrial cancer after estrogen and estrogen-progestin replacement. Cancer Causes Control *10*, 253-260.

Petaja-Repo, U. E., Hogue, M., Laperriere, A., Walker, P., and Bouvier, M. (2000). Export from the endoplasmic reticulum represents the limiting step in the maturation and cell surface expression of the human delta opioid receptor. J Biol Chem *275*, 13727-13736.

Pinter, J., and Thomas, P. (1995). Characterization of a progestogen receptor in the ovary of the spotted seatrout, Cynoscion nebulosus. Biol Reprod *52*, 667-675.

Pinter, J., and Thomas, P. (1997). The ovarian progestogen receptor in the spotted seatrout, *Cynoscion nebulosus*, demonstrates steroid specificity different from the progesterone receptors in other vertebrates. J Steroid Biochem Molec Biol *60*, 113-119.

Pinter, J., and Thomas, P. (1999). Induction of ovulation of mature oocytes by the maturation-inducing steroid 17,20β,21-trihydroxy-4-pregnen-3-one in the spotted seatrout. Gen Comp Endocrinol *115*, 200-209.

Porter, A., and Jänicke, R. (1999). Emerging roles of caspase-3 in apoptosis. Cell Death Diff *6*, 99-104.

Qiu, M., Olson, A., Faivre, E., Horwitz, K. B., and Lange, C. (2003). Mitogen-activated protein kinase regulates nuclear association of human progesterone receptors. Mol Endocrinol *17*, 628-642.

Quirk, S., Cowan, R., and Harman, R. (2004). Progesterone receptor and the cell cycle modulate apoptosis in granulosa cells. Endocrinology *145*, 5033-2043.

Rae, M. T., Menzies, G. S., and Bramley, T. A. (1998). Bovine ovarian non-genomic progesterone binding sites: presence in follicular and luteal cell membranes. J Endo *159*, 413-427.

Raza, F. S., Takemori, H., Tojo, H., Okamoto, M., and Vinson, G. P. (2001). Identification of the rat adrenal zona fasciculata/reticularis specific protein, inner zone antigen (IZAg), as the putative membrane progesterone receptor. Eur J Biochem *268*, 2141-2147.

Reiner, A., Reiner, G., Spona, J., Schemper, M., and Holzner, J. H. (1988). Histopathologic characterization of human breast cancer in correlation with estrogen receptor status. A comparison of immunocytochemical and biochemical analysis. Cancer *61*, 1149-1154.

Revelii, A., Massobria, M., and Tesarik, J. (1998). Nongenomic actions of steroid hormones in reproductive tissues. Endocr Rev *19*, 3-17.

Richer, J. K., Lange, C. A., Manning, N. G., Owen, G., Powell, R., and Horwitz, K. B. (1998). Convergence of progesterone with growth factor and cytokine signaling in

breast cancer. Progesterone receptors regulate signal transducers and activators of transcription expression and activity. J Biol Chem *273*, 31317-31326.

Rodway, M. R., Swan, C. L., Crellin, N. K., Gillio-Meina, C., and Chedrese, P. J. (1999a). Steroid regulation of progesterone synthesis in a stable porcine granulosa cell line: a role for progestins. J Steroid Biochem Mol Biol *68*, 173-180.

Rodway, M. R., Swan, C. L., Gillio-Meina, C., Crellin, N. K., Flood, P. F., and Chedrese, P. J. (1999b). Regulation of steroidogenesis in jc-410, a stable cell line of porcine granulosa origin. Mol Cell Endocrinol *148*, 87-94.

Ross, R. K., Paganini-Hill, A., Wan, P. C., and Pike, M. C. (2000). Effect of hormone replacement therapy on breast cancer risk: estrogen versus estrogen plus progestin. J Natl Cancer Inst *92*, 328-332.

Rueda, B., Hendry, I., Hendry, W., Stormshak, F., Slayden, O., and Davis, J. (2000). Decreased progesterone levels and progesterone receptor antagonists promote apoptotic cell death in bovine luteal cells. Bio Rep *62*, 269-276.

Runko, E., Wideman, C., and Kaprielian, Z. (1999). Cloning and expression of VEMA: a novel ventral midline antigen in the rat CNS. Mol Cell Neurosci *14*, 428-443.

Schairer, C., Lubin, J., Troisi, R., Sturgeon, S., Brinton, L., and Hoover, R. (2000). Estrogen-progestin replacement and risk of breast cancer. Jama *284*, 691-694.

Schreiber, J. R., Nakamura, K., and Erickson, G. F. (1981). Progestins inhibit FSH-stimulated granulosa estrogen production at a post-cAMP site. Mol Cell Endocrinol *21*, 161-170.

Scott, A. P., Sheldrick, E. L., and Flint, A. P. F. (1982). Measurement of 17α,20β-dihydroxy-4-pregnen-3-one in plasma of trout (*Salmo gairdneri* Richardson):

seasonal changes and response to salmon pituitary extract. Gen Comp Endocrinol *46*, 444-451.

Selmin, O., Lucier, G. W., Clark, G. C., Tritscher, A. M., Vanden Heuvel, J. P., Gastel, J. A., Walker, N. J., Sutter, T. R., and Bell, D. A. (1996). Isolation and characterization of a novel gene induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin in rat liver. Carcinogenesis *17*, 2609-2615.

Shaw, J. A., Udokang, K., Mosquera, J. M., Chauhan, H., Jones, J. L., and Walker, R. A. (2002). Oestrogen receptors alpha and beta differ in normal human breast and breast carcinomas. J Pathol *198*, 450-457.

She, Q. B., Ma, W. Y., Zhong, S., and Dong, Z. (2002). Activation of JNK1, RSK2, and MSK1 is involved in serine 112 phosphorylation of Bad by ultraviolet B radiation. J Biol Chem *277*, 24039-24048.

Shupnik, M. A. (2007). Estrogen receptor-beta: why may it influence clinical outcome in estrogen receptor-alpha positive breast cancer? Breast Cancer Res *9*, 107.

Shyamala, G. (1999). Progesterone signaling and mammary gland morphogenesis. J Mammary Gland Biol *4*, 89-104.

Shyamala, G., Yang, X., Cardiff, R. D., and Dale, E. (2000). Impact of progesterone receptor on cell-fate decisions during mammary gland development. Proc Natl Acad Sci U S A *97*, 3044-3049.

Skildum, A., Faivre, E., and Lange, C. A. (2005). Progesterone receptors induce cell cycle progression via activation of mitogen-activated protein kinases. Mol Endocrinol *19*, 327-339.

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science *235*, 177-182.

Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and et al. (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science *244*, 707-712.

Song, G., Ouyang, G., and Bao, S. (2005). The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med *9*, 59-71.

Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S.*, et al.* (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A *98*, 10869-10874.

Su, L., Morgan, P. R., and Lane, E. B. (1996). Expression of cytokeratin messenger RNA versus protein in the normal mammary gland and in breast cancer. Hum Pathol *27*, 800-806.

Svensson, E. C., Markstrom, E., Andersson, M., and Billig, H. (2000). Progesterone receptor-mediated inhibition of apoptosis in granulosa cells isolated from rats treated with human chorionic gonadotropin. Biol Reprod *63*, 1457-1464.

Swan, C. L., Agostini, M. C., Bartlewski, P. M., Feyles, V., Urban, R. J., and Chedrese, P. J. (2002). Effects of progestins on progesterone synthesis in a stable porcine granulosa cell line: control of transcriptional activity of the cytochrome p450 side-chain cleavage gene. Biol Reprod *66*, 959-965.

Tang, Y. T., Hu, T., Arterburn, M., Boyle, B., Bright, J. M., Emtage, P. C., and Funk, W. D. (2005). PAQR proteins: a novel membrane receptor family defined by an ancient 7-transmembrane pass motif. J Mol Evol *61*, 372-380.

Telleria, C. M., Stocco, C. O., Stati, A. O., and Deis, R. P. (1999). Progesterone receptor is not required for progesterone action in the rat corpus luteum of pregnancy. Steroids *64*, 760-766.

Thomas, P., and Das, S. (1997). Correlation between binding affinities of C21 steroids for the maturation-inducing steroid membrane receptor in spotted seatrout ovaries and their agonist and antagonist activities in an oocyte maturation bioassay. Biol Reprod *57*, 999-1007.

Thomas, P., and Doughty, K. (2004). Disruption of rapid, nongenomic steroid actions by environmental chemicals: interference with progestin stimulation of sperm motility in Atlantic croaker. Environ Sci Technol *38*, 6328-6332.

Thomas, P., Pang, Y., Dong, J., Groenen, P., Kelder, J., de Vlieg, J., Zhu, Y., and Tubbs, C. (2007). Steroid and G protein binding characteristics of the seatrout and human progestin membrane receptor alpha subtypes and their evolutionary origins. Endocrinology *148*, 705-718.

Thomas, P., Pang, Y., Filardo, E. J., and Dong, J. (2005). Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology *146*, 624-632.

Thomas, P., and Trant, J. (1989). Evidence that 17α,20β,21-trihydroxy-4-pregnen-3-one is a maturation-inducing steroid in spotted seatrout. Fish Phys Biochem *7*, 185-191.

Tokumoto, M., Nagahama, Y., Thomas, P., and Tokumoto, T. (2006). Cloning and identification of a membrane progestin receptor in goldfish ovaries and evidence it is an intermediary in oocyte meiotic maturation. Gen Comp Endocrinol *145*, 101-108.

Trant, J., and Thomas, P. (1989). Isolation of a novel maturation-inducing steroid produced *in vitro* by ovaries of Atlantic croaker. Gen Comp Endo *75*, 397-404.

Tubbs, C. (2007) Mechanisms of progestin–stimulated sperm hypermotility in two teleosts: the Atlantic croaker (*Micropogonias undulatus*) and the southern flounder (*Platylicthys lethigstomata*), Ph. D., The University of Texas, Port Aransas.

Vallejo, G., Ballare, C., Baranao, J. L., Beato, M., and Saragueta, P. (2005). Progestin activation of nongenomic pathways via cross talk of progesterone receptor with estrogen receptor beta induces proliferation of endometrial stromal cells. Mol Endocrinol *19*, 3023-3037.

Van den Broeck, W., D'haeseleer, M., Coryn, M., and Simoens, P. (2002). Cell-specific distribution of progesterone receptors in the bovine ovary. Reprod Dom Anim *37*, 314-320.

Vares, G., Ory, K., Lectard, B., Levalois, C., Altmeyer-Morel, S., Chevillard, S., and Lebeau, J. (2004). Progesterone prevents radiation-induced apoptosis in breast cancer cells. Oncogene *23*, 4603-4613.

Varghese, C. (2007). The significance of oestrogen and progesterone receptors in breast cancer. J Clin Diag Res *3*, 198-203.

Vermeirsch, H., Simoens, P., Coryn, M., and Van den Broeck, W. (2001). Immunolocalization of progesterone receptors in the canine ovary and their relation to sex steroid hormone concentrations. Reproduction *122*, 73-83.

Visser, M., Jiwa, M., Horstman, A., Brink, A. A., Pol, R. P., Diest, P. V., Snijders, P. J., and Meijer, C. J. (2008). Intra-operative rapid diagnostic method based on CK19 mRNA expression for the detection of lymph node metastases in breast cancer. Int J Cancer.

von Maillot, K., Horke, W., and Prestele, H. (1982). Prognostic significance of the steroid receptor content in primary breast cancer. Arch Gynecol *231*, 185-190.

Wehling, M. (1997). Specific, nongenomic actions of steroid hormones. Annu Rev Physiol *59*, 365-393.

Wright, C., Nicholson, S., Angus, B., Sainsbury, J. R., Farndon, J., Cairns, J., Harris, A. L., and Horne, C. H. (1992). Relationship between c-erbB-2 protein product expression and response to endocrine therapy in advanced breast cancer. Br J Cancer *65*, 118-121.

Xu, R., Feiner, H., Li, P., Yee, H., Inghirami, G., Delgado, Y., and Perle, M. A. (2003). Differential amplification and overexpression of HER-2/neu, p53, MIB1, and estrogen receptor/progesterone receptor among medullary carcinoma, atypical medullary carcinoma, and high-grade invasive ductal carcinoma of breast. Arch Pathol Lab Med *127*, 1458-1464.

Zhu, Y., Bond, J., and Thomas, P. (2003a). Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. Proc Natl Acad Sci U S A *100*, 2237-2242.

Zhu, Y., Rice, C. D., Pang, Y., Pace, M., and Thomas, P. (2003b). Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc Natl Acad Sci U S A *100*, 2231-2236.

Vita

Gwen Ellen Dressing was born on October 17, 1980 in Pittsburgh, Pennsylvania to Robert James and Joanne Myrtle Dressing. Following graduation from Thomas Jefferson High School she entered Gettysburg College in August of 1998. She spent August to December of 2000 at James Cook University in Townsville, Queensland Australia prior to being awarded a Bachelor of Science degree in Biology and Environmental Studies from Gettysburg College in 2002. She entered the Graduate School of the University of Texas at Austin in August of 2002.